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**TITLE: INVOLVEMENT OF LIPID METABOLISM IN THE ACTION OF  
PHOSPHOLIPASE A<sub>2</sub> NEUROTOXINS**

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Presynaptically-acting neurotoxins (PSNTXs) from snake venom irreversibly stimulate acetylcholine (ACh) release from and inhibit choline uptake into synaptosomes, with rat synaptosomes affected by more toxins than those from mice. The inclusion of BSA in the incubation medium at the time of toxin exposure has allowed a clear distinction to be made between nonPSNTX phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) and the PSNTXs in synaptosome preparations. The stimulation of ACh release by scutoxin and pseudexin, but not by  $\beta$ -bungarotoxin ( $\beta$ -Butx), was antagonized only by *neutralizing* antibodies to pseudexin. The PSNTXs exhibit a pattern of phospholipid hydrolysis different from nonPSNTX PLA<sub>2</sub>s, as determined either by type of fatty acid liberated, or by the type of phospholipid hydrolyzed. This PLA<sub>2</sub> activity may play a role in neurotoxicity. PSNTXs ( $\beta$ -Butx), cardiotoxins (CTXs), melittin and myotoxin *a* all cause Ca<sup>2+</sup> release from the terminal cisternae of the sarcoplasmic reticulum through the Ca<sup>2+</sup> release channel. Melittin and CTX, but not PSNTXs or myotoxin *a*, activate phospholipase C activity with no requirement for extracellular Ca<sup>2+</sup>.

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## INTRODUCTION

### **Abbreviations**

ACh, acetylcholine; p-BPB, *p*-bromophenacyl bromide; BSA, bovine serum albumin; BthTx, bothropstoxin (myotoxin) from *Bothrops jararacussa* venom;  $\beta$ -Butx,  $\beta$ -bungarotoxin; Ch, choline; CHE, cholesterol esters; CL, cardiolipin; CTX, cardiotoxin; DG, diacylglyceride; FA, fatty acid; FFA, free fatty acid; GC, gas chromatography; HPLC, high-performance (or high-pressure) liquid chromatography; HSRF, heavy sarcoplasmic reticulum fraction; LPC, lysophosphatidylcholine; NL, neutral lipid; nonPSNTX PLA<sub>2</sub>, nonpresynaptically-acting phospholipase A<sub>2</sub>; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLB, phospholipase B; PLC, phospholipase C; PS, phosphatidylserine; PSNTX, presynaptically-acting PLA<sub>2</sub> neurotoxin; SM, sphingomyelin; TG, triacylglyceride; TCICR, threshold of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release; TLC, thin-layer chromatography.

### **Nature of the Problem**

A number of potent (LD<sub>50</sub> 1-50  $\mu$ g/kg) presynaptically-acting neurotoxins (PSNTXs) have been isolated from snake venoms, all having phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity. Very little is known about the mechanism(s) of action of these agents and the role of PLA<sub>2</sub> activity in altering acetylcholine (ACh) release and choline (Ch) uptake. In recent years it has become recognized that these toxins are potential biological warfare agents. Snake venoms also contain smaller molecular weight cardiotoxins (CTXs) that greatly potentiate phospholipid (PL) hydrolysis by PLA<sub>2</sub> enzymes, allowing penetration of these enzymes to formerly inaccessible substrates. Understanding the modes of action of these toxins would aid the development of effective therapeutic and prophylactic approaches. This contract examines: 1) the action of PSNTXs and CTXs on ACh release and Ch uptake; 2) the role of PLA<sub>2</sub> activity in toxin action; 3) the action of PSNTXs and CTXs on Ca<sup>2+</sup> regulation; and; 4) effects of the toxins on virtually all aspects of endogenous (tissue) lipid metabolism. The three hypotheses tested were: 1) the potency of the presynaptic neurotoxins is due to the expression of PLA<sub>2</sub> activity on the inner leaflet of the plasma membrane bilayer at a very crucial target site; 2) the CTXs activate endogenous PLA<sub>2</sub> activity; and 3) PSNTXs and CTXs alter Ca<sup>2+</sup> regulation in cells, either as a direct action or secondary to alterations in lipid metabolism.

### **Background of Previous Work (Updated from Mid-Term Report in some areas)**

PSNTXs from snake venoms vary considerably in size (ca. 13.5-80 kD; Howard, 1982; Chang, 1985; Harris, 1985). The site of action of snake venom PLA<sub>2</sub> neurotoxins was first identified to be the neuromuscular junction for  $\beta$ -bungarotoxin ( $\beta$ -Butx) - the prototypic model for this group of toxins (Chang and Lee, 1963). The cause of death following envenomation with these toxins is respiratory failure. The PLA<sub>2</sub> toxins cause a brief initial decrease in transmitter release, followed by greatly enhanced and, ultimately, completely abolished release (Howard, 1982; Chang, 1985; Harris, 1985).

The PSNTXs can be either single chain toxins or consist of two to four subunits (Chang, 1985; Harris, 1985). The majority of these toxins are basic, or have at least one basic subunit. The basic portion of the molecule appears to be the toxic unit and contains the PLA<sub>2</sub> activity (Harris, 1985). The binding sites of the PSNTXs are most likely not identical (Chang

and Su, 1980; Lambeau et al., 1989). The toxins do not appear to leave the plasma membrane once bound (Trivedi et al., 1989; Simpson et al., 1993). While the PSNTXs do not appear to be internalized, they do become inaccessible to antibodies (Simpson et al., 1993), suggesting the active site may reach into the inner leaflet of the bilayer.

While the exact mechanism of action of PSNTXs is unknown, recent evidence has suggested that these toxins may block certain  $K^+$  channels that are slowly activating (Dreyer and Penner, 1987) or voltage-dependent (Rowan and Harvey, 1988). Other consequences of the toxin action appears to be elevated cytoplasmic  $Ca^{2+}$  levels (Howard and Gundersen, 1980) and depolarization of nerve terminals (Sen and Cooper, 1978). The toxins also uncouple mitochondrial respiration (Howard, 1975; Ng and Howard, 1980; Rugolo et al., 1986). These effects could be attributed to free fatty acids (FFAs). A differentiation between the action of  $\beta$ -Butx on the plasma membrane and that on mitochondria has been observed that is dependent on the presence of bovine serum albumin (BSA), a scavenger of fatty acids, in the bathing medium (Rugolo et al., 1986).

The CTXs are, for the most part, small molecular weight (ca. 7 kD) polypeptides that induce arrhythmias and eventually cardiac arrest (Lee et al., 1968; Condrea, 1974; 1979). The CTXs are active on most membranes and can induce contractures in skeletal muscle, hemolysis of erythrocytes and lysis of cell cultures. These agents work in mutual synergy with  $PLA_2$  enzymes, the  $PLA_2$  activity greatly enhancing the hemolytic activity of CTX and the action of CTX greatly enhancing the penetrating ability, and consequently the hydrolytic activity, of the  $PLA_2$  (Condrea, 1974; 1979). Melittin has many of the same characteristics as CTXs. We have recently demonstrated that melittin allows bee venom  $PLA_2$  access to the inner leaflet of artificial membrane bilayers (Fletcher et al., 1990a). CTX has also been reported to activate endogenous  $PLA_2$  enzyme activity (Shier, 1979).

All PSNTXs from snake venoms have  $PLA_2$  activity. High levels of lipolysis will certainly alter neurotransmitter release in a nonspecific manner. Indeed, addition of a nonneurotoxic  $PLA_2$  enzyme to synaptosomes does result in enhanced (Sen et al., 1976; Fletcher and Middlebrook, 1986) and, ultimately, inhibited (Fletcher and Middlebrook, 1986) transmitter release. The greater potency of  $\beta$ -Butx relative to a less toxic  $PLA_2$  enzyme in causing these effects (Fletcher and Middlebrook, 1986) may simply relate to a greater specific binding to a crucial site and PL hydrolysis on the inner leaflet of the bilayer. The toxin-induced inhibition of neurotransmitter release is likely due to inhibition of choline (Ch) uptake (Sen et al., 1976; Fletcher and Middlebrook, 1986). Low, barely detectable levels of hydrolysis are more relevant to upsetting a physiological mechanism of lipolysis associated with neurotransmitter release. In such a mechanism a highly localized change in the ratio of saturated to unsaturated FFAs may significantly alter neurotransmitter release. Unsaturated FFAs have effects on the uptake of Ch and amino acids and the release of ACh that mimic treatment with  $PLA_2$  (Rhoads et al., 1983; Boksa et al., 1988). In contrast, saturated FFAs, which predominate in the membrane in the absence of lipolytic activity, have no effect on transmitter uptake or ACh release (Rhoads et al., 1983; Boksa et al., 1988).

Considerable controversy exists regarding the role of  $PLA_2$  activity in the action of a number of toxin classes, including neurotoxic and cardiotoxic  $PLA_2$  agents. The confusion regarding the role of  $PLA_2$  activity in neurotoxin action is best expressed in a recent review (Harris, 1985). The  $PLA_2$  activity of the PSNTXs is extremely low compared to other venom



PLA<sub>2</sub> enzymes, and is most often only observed on purified PL substrates in the presence of charged detergent substrates (Strong et al., 1976; Radvanyi et al., 1987). In general, most laboratories agree that there is a mechanism in addition to PLA<sub>2</sub> activity that plays a role in the toxic action of these agents. Certainly there is a binding component rendering specificity to these toxins that is active under conditions that (most likely) eliminate much of the enzyme activity. This binding action has been suggested to account for the initial depression of ACh release (Abe et al., 1977; Abe and Miledi, 1978; Livengood et al., 1978; Kelly et al., 1979; Caratsch et al., 1981; 1985; Halliwell et al., 1982).

Several laboratories have suggested that PLA<sub>2</sub> activity plays a crucial role in the latter two phases of toxin action (Strong et al., 1976; Abe et al., 1977; Chang et al., 1977; Abe and Miledi, 1978; Livengood et al., 1978; Kelly et al., 1979; Halliwell et al., 1982; Hawgood and Smith, 1989). While some evidence suggests that PLA<sub>2</sub> activity plays no role in the toxic action (Rosenberg et al., 1989), these studies are all inconclusive, as no study to date has directly examined the PLA<sub>2</sub> activity of these neurotoxins on the biological substrates affected. For example, replacement of Ca<sup>2+</sup> by Sr<sup>2+</sup> is presumed to eliminate PLA<sub>2</sub> activity; however, a surprisingly high level of PLA<sub>2</sub> activity is maintained under this condition (Fletcher et al., 1981; Ghassemi et al., 1988). Even if PLA<sub>2</sub> activity were determined on biological substrates, the methods employed by investigators in the field most likely would not be sensitive, specific or thorough enough to yield conclusive results. In two cases in which FA production was determined by GC analysis (Ng and Howard, 1978; Noremborg and Parsons, 1986), the values reported were unrealistic.

Studies extrapolating results of PL hydrolysis on purified substrates to the action of toxins on biological substrates are meaningless. Chemical modification of a PLA<sub>2</sub> neurotoxin can greatly affect the enzymatic activity of the toxin, but often this only occurs toward one or a very few molecular species of PL. For example, the -COOH-C modification of  $\beta$ -BuTX decreased enzymatic activity by 50% toward an egg yolk substrate, but had no effect on hydrolysis of a lecithin substrate (Rosenberg et al., 1989). Lethality was not affected. Interpreting the findings with egg yolk as dissociating enzymatic activity from pharmacological activity is obviously incorrect. In our current studies some CTX fractions completely devoid of PLA<sub>2</sub> activity on purified substrates exhibit very significant levels of PL hydrolysis on biological substrates (red blood cells or cell cultures), when gas chromatographic (GC) analysis of FFAs is employed.

Since different FFAs have different effects on transmitter release (Boksa et al., 1988), it is essential to employ a method such as GC, so that the individual FFAs can be quantitated and shifts in the ratio of saturated to unsaturated FFAs can be monitored. In the snake venom PLA<sub>2</sub> literature the emphasis has been on subclasses of PL (phosphatidylcholine, etc.) hydrolyzed, not molecular species as evidenced in a recent review (Harris, 1985). The existence of reacylating enzymes in tissues that "restore" the hydrolyzed PL by attaching another FFA on to it would mean PLs will appear not to have been hydrolyzed at levels of PLA<sub>2</sub> activity less than the tissue reacylation activity. Using GC analysis of FFAs will detect even these low levels of PLA<sub>2</sub> activity. Radiolabeling cell cultures allows examination of: 1) the glycerol moiety; 2) FFAs or esters, and; 3) the phosphorylated bases of PLs. Therefore we can distinguish acylation reactions from alterations of head groups.

## Purpose of the Present Work

The present study tests three major hypotheses:

1. PSNTXs exert their effects on ACh release and Ch uptake by hydrolyzing specific PLs at crucial sites, perhaps on the inner leaflet of the plasma membrane bilayer
2. PSNTXs and CTXs activate endogenous processes of lipid metabolism
3. PSNTXs and CTXs alter  $\text{Ca}^{2+}$  regulation in cells

## Methods of Approach

**Objective #1.** *Compare the potencies and efficacies of PSNTXs as enhancers and ultimately inhibitors of ACh release from synaptosomes and rat PC12 cell and the mouse NB41A3 mouse neuroblastoma cell lines.* The relative orders of potency of a series of  $\text{PLA}_2$  neurotoxins on Ch uptake and ACh release were determined in mouse brain synaptosomes and in differentiated and nondifferentiated cell lines.

We have examined:

- 1) The relative potencies ( $\text{LD}_{50}$ s) of the toxins *in vivo*
- 2) Choline uptake ( $[^14\text{C}]$ choline) into synaptosomes
  - a) the time course of choline uptake
  - b) the effects of temperature on choline uptake
  - c) the kinetics ( $K_m$ ,  $V_{max}$ ) of choline uptake
  - d) the dose-response relationships as regards inhibition of choline uptake by PSNTXs and nonPSNTX  $\text{PLA}_2$ s
  - e) the effects of coincubation with BSA (to extract the FAs produced) on the action of the PSNTXs
  - f) involvement of acidic and basic subunits in inhibition of choline uptake (Mojave toxin model)
  - g) the effects of PSNTXs on  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -independent choline uptake
  - h) reversibility of toxin action
  - i) compare rat to mouse synaptosomes
- 3) Choline uptake ( $[^14\text{C}]$ choline) into PC12 and NB41A3 cell lines
- 4) Acetylcholine release ( $[^14\text{C}]$ ) from synaptosomes
  - a) evaluation of a TLC method for separation of ACh and Ch and comparison to a tetraphenylboron/3-heptanone-based extraction for determination of ACh
  - b) comparison of radiolabeled ACh release to ACh release determined by HPLC
  - c) the dose-response relationships as regards stimulation of ACh release by PSNTXs and two non PSNTX  $\text{PLA}_2$
  - d) effects of coincubation with BSA on the action of PSNTXs
  - e) effects of temperature on toxin-induced ACh release
  - f) time course of toxin action
  - g) reversibility of toxin action
  - h) comparison of toxin action on rat vs. mouse brain synaptosomes
  - i) effects of increasing concentration of BSA on toxin action
  - j) effects of antibodies (neutralizing and nonneutralizing) to pseudexin on pseudexin-induced ACh release and cross-reactivity with other PSNTXs
  - j) compare rat to mouse synaptosomes

- 5) Acetylcholine release ( $[^{14}\text{C}]$ ) from PC12 and NB41A3 cell lines
- 6) Effects of a synthetic peptide from *Trimeresurus wagleri* snake venom on the phrenic nerve-diaphragm preparation

**Objective #2.** *Examine the interaction between snake venom PSNTXs and CTXs.*

No studies were conducted in relation to this objective. The primary reason for this was that J.O. Schmidt (Arizona) was conducting similar studies and was not observing any enhancement of activity of PSNTX activity by melittin using *in vivo* administration of toxins (personal communications). Greater emphasis was placed on the lipid metabolism studies (repeat of GC analysis in synaptosomes at a higher BSA concentration) to compensate for omission of this objective.

**Objective #3.** *Examine the role of PLA<sub>2</sub> activity in PSNTXs and CTX action.* This is the first study to directly determine all substrates and products of PLA<sub>2</sub> activity (PLs, lysoPLs and FFAs) on the biological substrate affected by the toxins.

The role of PLA<sub>2</sub> activity in the action of the PSNTXs and CTXs has been examined in several ways:

- 1) Effects of PSNTXs on lipid metabolism in cell culture systems in which the lipids were preradiolabeled
  - a) determine whether toxins activate endogenous lipases
  - b) determine phospholipid substrate preferences in differentiated and nondifferentiated nerve and muscle cells
  - c) determine the effects of radiolabeling with saturated or unsaturated fatty acid on phospholipid hydrolysis
  - d) determine effects of BSA on overall enzymatic activity and substrate preference
- 2) Effects of PSNTXs on FFA production in synaptosomes, determined by GC analysis
  - a) determined types of fatty acids release by the PSNTXs vs. nonPSNTX PLA<sub>2</sub>s
  - b) determine "extractability" of FAs by BSA as a measure of external vs. internal production
- 3) Effects of CTXs, melittin and myotoxins on lipid metabolism in cell culture systems in which the lipids were preradiolabeled
  - a) effects of *p*-BPB-treatment on phospholipid hydrolysis by CTXs, melittin and myotoxins (to abolish PLA<sub>2</sub> activity associated with any venom PLA<sub>2</sub> contamination)
  - b) effects of toxins on liberation of radiolabeled fatty acid-associated products (primarily diacylglycerol, triglyceride and phospholipids)
  - c) determine time course of PLA<sub>2</sub> activity

**Objective #4.** *Correlation of effects on ACh release and FFA release (lipid metabolism) with those on cytoplasmic Ca<sup>2+</sup>.* The effects of PSNTXs and CTXs were examined on Ca<sup>2+</sup> release from terminal cisternae preparations.

The involvement of Ca<sup>2+</sup> regulation in stimulation of transmitter release (PSNTXs) or contracture induction (CTXs and related toxins), either directly or as a consequence of altered lipid metabolism was examined in the following manner:

- 1) Effects of a PSNTX ( $\beta$ -Butx) on the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (TCICR) in heavy sarcoplasmic reticulum containing fractions
  - a) dependence on  $\text{Ca}^{2+}$  preload
  - b) association of  $\text{Ca}^{2+}$  release with the ryanodine receptor (  $\text{Ca}^{2+}$  release channel)
- 2) Effects of CTXs and related toxins (melittin and myotoxin *a*) on the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (TCICR) in heavy sarcoplasmic reticulum containing fractions
  - a) dependence on  $\text{Ca}^{2+}$  preload
  - b) association of  $\text{Ca}^{2+}$  release with the ryanodine receptor (  $\text{Ca}^{2+}$  release channel)
  - c) effects of toxins on the binding of ryanodine to its receptor
  - d) determine involvement of  $\text{Ca}^{2+}$  uptake in the action of myotoxin *a*
  - e) confirm contracture induction in skeletal muscle by all toxins examined
  - f) determine if  $\text{PLA}_2$ -free fractions (*p*-BPB-treated) and/or synthetic toxins have the same activity as the native fraction

**Objective #5.** *Examine the effects of PSNTXs and CTXs on lipid metabolism.* The effects of the PSNTXs and CTXs were examined on skeletal muscle cultures in which the lipids are preradiolabeled on the head groups, glycerol backbone and fatty esters.

The effects of the PSNTXs and CTXs on lipid metabolism were examined in the following manner:

- 1) Effects of PSNTXs on lipid metabolism in cell culture systems in which the lipids were preradiolabeled
  - a) determine whether toxins activate endogenous lipases
  - b) determine phospholipid substrate preferences in differentiated and nondifferentiated nerve and muscle cells
  - c) determine effects of BSA on overall enzymatic activity and substrate preference
- 2) Effects of CTXs, melittin and myotoxins on lipid metabolism in cell culture systems in which the lipids were preradiolabeled
  - a) effects of *p*-BPB-treatment on phospholipid hydrolysis by CTXs, melittin and myotoxins (to abolish  $\text{PLA}_2$  activity associated with any venom  $\text{PLA}_2$  contamination)
  - b) effects of toxins on liberation of radiolabeled fatty acid-associated products (primarily diacylglycerol, triglyceride and phospholipids)
  - c) effects of toxins on liberation of specific products of PLC and PLD activity (phosphoethanolamine, phosphocholine, ethanolamine and choline)
  - d) determine calcium dependence of activation of tissue lipases
  - e) determine time course of toxin action
  - f) determine activation of tissue lipases in several types of cell cultures and under various states of cell differentiation
  - g) determine the effects of pertussis and cholera toxin on activation of tissue lipases

**Objective #6.** *Effects of altered lipid environment on PSNTX action.*

The lack of effect of the toxins on ACh release from these cell cultures caused us to use alternative methods for examining this objective.

- 1) Comparison of  $\text{PLA}_2$  activity of the PSNTXs on cells of various types

- a) compare effects on nerve to those on skeletal muscle
- b) compare effects on differentiated nerve cells to nondifferentiated nerve cells
- 2) Determine the effects on PLA<sub>2</sub> activity of the PSNTXs when the lipid environment is altered by incubation with two different types of fatty acid (18:2 and 18:0)

**Objective #7. Study penetration to inner layer of bilayer.**

- 1) Effects of PSNTXs on lipid metabolism in cell culture systems in which the lipids were preradiolabeled
  - a) determined phospholipid substrate preferences in cell cultures to determine if intracellular phospholipids were preferentially hydrolyzed
  - b) determine effects of BSA on overall enzymatic activity and substrate preference, since BSA extracts FAs from the extracellular leaflet of the plasma membrane bilayer
- 2) Effects of PSNTXs on FFA production in synaptosomes, determined by GC analysis
  - a) determined types of fatty acids release by the PSNTXs vs. nonPSNTX PLA<sub>2</sub>s
  - b) determine "extractability" of FAs by BSA as a measure of external vs. internal production
- 3) Effects of CTXs, melittin and myotoxins on lipid metabolism in cell culture systems
  - a) determine extracellular calcium dependence of activation of tissue lipases to examine if plasma membrane or intracellular site is involved in toxin action

## **BODY**

### **Experimental Methods**

#### **Materials**

Scutoxin, taipoxin, the *Naja naja kaouthia* PLA<sub>2</sub> and crotoxin were obtained from Ventoxin Laboratories, Inc. (Frederick, MD). The *Naja naja atra* PLA<sub>2</sub> was a gift from Dr. Leonard A. Smith (Fort Detrick, MD). CTX from *Naja naja kaouthia* venom (Lot# 125F-4007), lipid standards, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Percoll, FA-free BSA, bee venom melittin,  $\beta$ -bungarotoxin, *p*-bromophenacyl bromide (*p*-BPB), phosphocreatine, creatine phosphokinase, Mg-ATP, 3-([cholamidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS), 1,4-piperazineethanesulfonic acid (PIPES), pepstatin, iodoacetamide, phenylmethylsulfonyl fluoride, leupeptin, benzamidine and arsenazo III were purchased from Sigma Chemical Company (St. Louis, MO). Morpholinopropane sulfonic acid (MOPS) was obtained from Calbiochem (San Diego, CA). Synthetic melittin from Peninsula Laboratories, Inc. (Belmont, CA). Bothropstoxin was obtained from Dr. Lea Rodrigues-Simioni (UNICAMP, Brazil). Mojave toxin (whole toxin and acidic and basic subunits) was obtained from Dr. Ivan Kaiser (University of Wyoming). Convulxin was obtained from Dr. Cassion Bon (Pasteur Inst., France). Myotoxins *a* was obtained from Dr. Charlotte Ownby (Oklahoma State University). [<sup>3</sup>H]Ryanodine (60 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). [<sup>14</sup>C]Choline, [<sup>14</sup>C]ethanolamine and [<sup>14</sup>C]fatty acids (50-60 mCi/mmol) were obtained from Amersham (Arlington Heights, IL). In some cases the CTX and melittin fractions were treated with *p*-BPB to inactivate contaminating

venom PLA<sub>2</sub> prior to use, as previously described (Jiang et al., 1989; Fletcher et al., 1991a).

#### I.V. LD<sub>50</sub> Determinations

Male Swiss-Webster mice (20-25 g) were restrained and injected in the tail vein with about 0.2 ml of toxin in saline solution. Since the LD<sub>50</sub> values have been published for all of these toxins, we focussed our analysis on those doses immediately above and below the published values. The number of animals surviving after 16 hr was used to estimate the LD<sub>50</sub>.

#### Isolation of Synaptosomes

An enriched synaptosomal fraction was prepared from mouse brain (Dunkley et al., 1988). Briefly, whole brain from six male Swiss Webster mice (20-30 g), or one male Wistar rat (200-300 g) was homogenized (3 g tissue per 9 ml) in sucrose (0.32 M), ethylenediaminetetraacetic acid (EDTA; 1 mM), dithiothreitol (0.25 mM), pH 7.4 (4°C). The homogenate was centrifuged (1,000 x g; 10 min) and the supernatant adjusted to 13 ml. The supernatant (4 mg protein/ml) was applied to a discontinuous Percoll gradient (2 ml each 23%, 15%, 10%, 3% Percoll; v/v; pH 7.4) and centrifuged at 32,500 x g for 15 min. Fractions 3 and 4 (Dunkley et al., 1988) were pooled for use in the ACh release and Ch uptake studies.

#### Choline Uptake

High-affinity choline uptake was determined by a modification of our previous study (Fletcher and Middlebrook, 1986), based on the original method of Yamamura and Snyder (Yamamura and Snyder, 1972). The pooled synaptosomal fractions were centrifuged (Beckman JA-20 rotor; 15,000 RPM; 15 min), the supernatant discarded and pellets resuspended in incubation buffer comprised of (in mM): HEPES 10, NaCl 137, KCl 2.7, CaCl<sub>2</sub> 1.7, MgCl<sub>2</sub> 0.7, D-glucose 20 and adjusted to pH 7.4. Aliquots of synaptosomes in 0.5 ml of incubation buffer were incubated with or without toxin and/or BSA 0.5% at 25°C (37°C when indicated) for 1 hr. The synaptosomes were centrifuged (Eppendorf 5414 microcentrifuge; 30 sec), the supernatant discarded and pellets washed, centrifuged and resuspended in incubation buffer. Aliquots of synaptosomes were added to tubes containing incubation buffer at 37°C and [<sup>14</sup>C]choline (2 μM). After a 4 min choline uptake period the incubates were filtered (Whatman GF/F filters) in a Hoefer (San Francisco, CA) FH224V filter holder and washed three times with 1 ml cold (4°C) incubation buffer. Scinti Verse™ II (Fisher Scientific Co.; Pittsburgh, PA) was used for liquid scintillation counting of radioactivity retained on the filters. In the case of Na<sup>+</sup>-free uptake medium, Tris-HCl (91 mM) was substituted for NaCl.

#### Acetylcholine Release

Acetylcholine release from synaptosomes was determined by a tetraphenylboron and 3-heptanone extraction technique, as previously described (Fletcher and Middlebrook, 1986). In brief, synaptosomes were preloaded with [<sup>14</sup>C]Ch to synthesize [<sup>14</sup>C]ACh in an incubation buffer comprised of (in mM): HEPES 10, NaCl 137, KCl 2.7, CaCl<sub>2</sub> 1.7, MgCl<sub>2</sub> 0.7, D-glucose 20 and adjusted to pH 7.4. Aliquots of synaptosomes in 0.5 ml of incubation buffer were incubated with or without toxin and/or BSA (0.5 or 1%) at 25°C (37°C when indicated)

for 30-60 min. A solution containing choline kinase was added to eliminate choline and to selectively extract ACh into tetraphenylboron and 3-heptanone. An aliquot of the organic phase was then evaporated and quantitated by liquid scintillation counting. A different methodology in which Ch and ACh in the aqueous phase were concentrated by lyophilization and then solubilized in a small volume, separated by TLC and then the radioactivity on the plate determined by a radioactivity scanner was also tested.

Acetylcholine release from cell lines was determined by preloading with [ $^{14}$ C]Ch for 16 hrs, then incubating with toxin. Choline kinase was added to the supernatant and the samples then extracted, as described above. In some cases the cells were differentiated with NGF (100  $\mu$ g/ml; 6 days; PC12) or sodium butyrate (0.5 mM; 5 days; NB41A3) prior to toxin addition.

#### Phrenic Nerve-Diaphragm Preparation

The mouse phrenic nerve-diaphragm was isolated and mounted, essentially as previously described for the rat preparation (Fletcher et al., 1981). The preparation was bathed in a modified Krebs solution at 37°C (Fletcher and Rosenberg, 1985). Preparations were adjusted for optimum length for twitch tension and equilibrated 1 hr before drug or toxin addition. Preparations were then either exposed to synthetic peptide toxin (1  $\mu$ M), or succinylcholine (50  $\mu$ M). Preparations exposed to succinylcholine were washed and reequilibrated for 10 min before exposure to synthetic peptide (1  $\mu$ M).

#### Radiolabeling and Analysis of Lipids in Cultured Cells

Primary cultures of human skeletal muscle were established from biopsies of vastus lateralis as previously described (Wieland et al., 1989). Ten to fourteen days (human and equine primary cultures), or 3-5 days (mouse C<sub>2</sub>C<sub>12</sub>, NB41A3 and rat PC12 cell lines) following addition of fusion-promoting medium (Wieland et al., 1989), radiolabeled FA ([ $^{14}$ C]linoleic acid; 10  $\mu$ M) and/or ethanolamine ([ $^{14}$ C]ethanolamine; 10  $\mu$ M), or choline ([ $^{14}$ C]choline; 10  $\mu$ M) was added to the culture medium for three days, as described previously for FAs in human and equine skeletal muscle cells (Fletcher et al., 1991a). In some cases the cells were differentiated with NGF (100  $\mu$ g/ml; 6 days; PC12) or sodium butyrate (0.5 mM; 5 days; NB41A3). Pertussis toxin (400 ng/ml) or cholera toxin (1000 ng/ml) were added to specified cultures 24 hrs prior to completion of labeling. Following incubation for 2 hrs with or without toxin at 37°C, the lipids were extracted and, for the FA studies, neutral and phospholipid components were separated by one-dimensional thin-layer chromatography (TLC) and the radioactivity associated with each phospholipid and neutral lipid component quantitated by a radioactivity scanner (Fletcher et al., 1990a; 1991a). For the ethanolamine or choline studies, the same lipid extraction was performed. However, the aqueous phase of the two-phase extraction containing the cell-associated phospholipid polar head groups was lyophilized, resoluted with methanol and the radioactivity in ethanolamine and phosphoethanolamine (or choline and phosphocholine) was separated by one-dimensional TLC on Analtech (Newark, DE) AVICEL F cellulose TLC plates (20 x 20 cm) using a solvent system comprised of n-butanol:methanol:acetic acid:ethylacetate:H<sub>2</sub>O [20:10:5:20:15 (Bluth et al., 1980)] and subsequent scanning of the TLC plate for radioactivity, as described above. The location of the radiolabel in phosphatidylethanolamine (CHCl<sub>3</sub> phase) was

confirmed by one-dimensional TLC, using the same phospholipid solvent system as used with the FAs.

#### **Extraction and Analysis of Lipids of Synaptosomes**

Synaptosomes were prepared and incubated with or without toxin and/or BSA for 1 hr, as described above. The incubates were centrifuged (Eppendorf 5414 microcentrifuge; 30 sec) and the lipids were extracted separately from the supernatant and pellets by the methods of Marinetti et al. (Marinetti et al., 1959) and Folch et al. (Folch et al., 1957), as previously described for red blood cells (Fletcher et al., 1987) and muscle (Fletcher et al., 1982; 1990b). The neutral lipids were separated by one-dimensional thin-layer chromatography (Fletcher et al., 1987), the FFAs methylated (Morrison and Smith, 1964) and the FA methyl esters separated on a Shimadzu (Columbia, MD) GC-9A gas chromatograph and quantitated using Beckman (Allendale, NJ) System Gold software, as previously described (Fletcher et al., 1987; 1990b). Heptadecanoic acid was added to the extract as an internal standard (Fletcher et al., 1987; 1990b). Protein was determined by a modification (Markwell et al., 1978) of the method of Lowry et al. (Lowry et al., 1951).

#### **Determination of Threshold of $\text{Ca}^{2+}$ -Induced $\text{Ca}^{2+}$ Release (TCICR)**

Heavy sarcoplasmic reticulum fractions were prepared by differential centrifugation (8,000 - 12,000 x g; Nelson, 1983) of homogenates of equine semimembranosus, human vastus lateralis, or porcine gracilis muscle. The TCICR was determined with pyrophosphate to increase the sensitivity of the assay (Palade, 1987a) and arsenazo III to detect  $\text{Ca}^{2+}$ , as previously described (Fletcher et al., 1990b; 1991b; c).  $\text{Ca}^{2+}$  was added in 10  $\mu\text{M}$  increments to 1.5 ml of a MOPS/KCl (pH 7.0) buffer containing 20-40  $\mu\text{g}$  protein, ATP and an ATP regenerating system maintained at 37°C (Palade, 1987a). CTX (10  $\mu\text{M}$ ),  $\beta$ -Butx (1 or 10  $\mu\text{M}$ ), melittin (0.1-1.0  $\mu\text{M}$ ) or myotoxin a (1-10  $\mu\text{M}$ ) was either added immediately after ATP-stimulated  $\text{Ca}^{2+}$  uptake had reached equilibrium, as previously described for the addition of FAs (Fletcher et al., 1990b), or after the indicated number of  $\text{Ca}^{2+}$  pulses (expressed as percent of the TCICR; Fletcher et al., 1991b).

#### **Ryanodine Binding**

For CTX and melittin studies, the ryanodine receptor was isolated by CHAPS solubilization from porcine longissimus dorsi by a modification of a procedure for use with skeletal muscle frozen and stored in liquid  $\text{N}_2$  (Valdivia et al., 1991b; Fletcher et al., 1992; 1993). The muscle was minced, thawed and then homogenized in a sucrose (0.3 M), HEPES-Tris buffer at pH 7.2 containing the following protease inhibitors: pepstatin, iodoacetamide, phenylmethylsulfonyl fluoride, leupeptin and benzamidine (Valdivia et al., 1991b). The supernatant from the first centrifugation step (4,000 x  $g_{\text{max}}$ ; 20 min; Beckman JA-20 rotor) was transferred and centrifuged (16,000 x  $g_{\text{max}}$ ; 20 min Beckman JA-20 rotor) a second time. The pellet from the second centrifugation step was resuspended in 4 ml of homogenizing buffer and diluted with 4 ml of CHAPS (1%) NaCl (1 M) and Tris maleate (40 mM) at pH 7.2. The preparations were incubated for 40 min at 4°C, centrifuged (60,000 x  $g_{\text{max}}$ ; 40 min; Beckman Type 40 rotor) and the supernatant, containing solubilized ryanodine receptor, was used for binding studies. Competitive binding of the toxins for the ryanodine receptor was



determined as described for a scorpion venom (Valdivia et al., 1991a). Ryanodine receptor preparations were incubated with a fixed concentration of [ $^3$ H]ryanodine (8 nM) and various concentrations (0.01-10  $\mu$ M) of CTX or melittin for 90 min at 37°C by a 1:6.25 dilution of CHAPS-solubilized protein into incubation buffer containing 0.2 M KCl, 1 mM Na<sub>2</sub>EGTA, 0.995 mM CaCl<sub>2</sub>, 10 mM Na-PIPES adjusted to pH 7.2. The concentration of protein was 60  $\mu$ g/ml and the concentration of free Ca<sup>2+</sup> was calculated to be 10  $\mu$ M (Valdivia et al., 1991b). Incubates were filtered on Whatman GF/B, the filters washed with cold incubation buffer and the radioactivity associated with the filters determined using liquid scintillation techniques (Vita et al., 1991). Nonspecific binding was determined in the presence of 10  $\mu$ M unlabeled ryanodine.

For myotoxin *a*, [ $^3$ H]ryanodine binding was performed on the HSR fraction isolated in the same manner as described above for the TCICR studies (8,000-12,000  $\times$  g), from fresh or liquid N<sub>2</sub> frozen equine semimembranosus muscle, as previously described (Yudkowsky et al., 1994). The assay was carried out in 0.1 ml KMOPS buffer containing HSR protein (2-5 mg/ml). Ethanol was evaporated from [ $^3$ H] ryanodine with nitrogen, and the alkaloid solubilized in binding buffer. Non specific binding was determined by the addition of an excess of unlabeled ryanodine (10  $\mu$ M). Suspensions of HSR were incubated with or without myotoxin *a* (10  $\mu$ M) for 90 minutes at 37°C. Samples were run in triplicate, filtered on glass fiber filters (Whatman GF/F) presoaked for 15 minutes in 5% polyethylenimine, and washed twice with 5 ml cold water. Filters were placed Scintiverse II cocktail overnight and counted on a Pharmacia (Piscataway, NJ) 1209 RackBeta liquid scintillation counter. The data were analyzed by Scatchard plots with the best fit line generated using SigmaPlot (Jandel Scientific; San Rafael, CA).

## Results - Presynaptically-Acting Phospholipase A<sub>2</sub> Neurotoxins

### Relative Potencies of the Toxins In Vivo

To compare the relative potencies of the toxins in vivo to those in vitro we first established mouse i.v. LD<sub>50</sub> values for several toxin fractions in our possession. These include: crotoxin (70  $\mu$ g/kg);  $\beta$ -Butx (40  $\mu$ g/kg); taipoxin (< 7  $\mu$ g/kg); *Naja naja atra* PLA<sub>2</sub> (8,600  $\mu$ g/kg); and scutoxin (40  $\mu$ g/kg). Pseudexin (ca. 1000  $\mu$ g/kg) was tested and provided by Dr. John L. Middlebrook (USAMRIID). Interestingly, we initially examined i.p. LD<sub>50</sub> values for  $\beta$ -Butx and taipoxin and found the value for  $\beta$ -Butx to be about the same as for i.v. administration, but the toxicity of taipoxin was reduced to below that of  $\beta$ -Butx.

### Choline Uptake

The time course of <sup>14</sup>C-choline (2  $\mu$ M) uptake at 37°C was linear for about 5 min (see Figure 1 in Mid-Term Report). Analysis by Lineweaver-Burk plot, yielding a single high affinity choline uptake process with a K<sub>m</sub> of 4  $\mu$ M (37°C, 4 min; see Figure 2 in Mid-Term Report). The viability of the choline uptake process in synaptosomes is retained considerably longer at 25°C than at 37°C (see Figure 3 in Mid-Term Report).

The dose-response relationships were examined as regards inhibition of choline uptake in the absence or presence of BSA for six PLA<sub>2</sub>s or PSNTXs (see Figure 4 of Mid-Term Report). Albumin, which is in blood, has complex effects on PLA<sub>2</sub>s. BSA binds the FFAs generated by PLA<sub>2</sub> activity and this can enhance, inhibit, or have no effect on PLA<sub>2</sub> activity

depending on the specific enzymes (Pluckthun and Dennis, 1985). In brief, BSA dramatically antagonized a nonPSNTX PLA<sub>2</sub> from *Naja naja atra* venom and has little or no effect on the four PSNTXs ( $\beta$ -BuTX, crotoxin, scutoxin, pseudexin) most active on mouse brain synaptosomes, as regards choline uptake (see Figure 4 of Mid-Term Report). We examined the effect of pseudexin on choline uptake that was not Na<sup>+</sup> dependent. The Na<sup>+</sup>-independent (nonspecific) choline uptake process that is not coupled to ACh synthesis and release was greatly inhibited by pseudexin, even with BSA in the medium (see Figure 4 of Mid-Term Report). This latter finding supports an action independent of nonspecific phospholipid hydrolysis.

Incubating the synaptosomes with the toxins for a brief period (1 min), washing the preparation free of unbound toxin and continuing the incubation for a total of 60 min (Figure 1) did not decrease the percentage of choline uptake inhibition by  $\beta$ -BuTX and pseudexin and these findings were basically independent of the presence of BSA (Figure 2). Pseudexin inhibition of Ch uptake seemed slightly dependent on the presence of the toxin for the entire 60 min (Figure 2). Therefore, most of the effects of the toxins on Ch uptake are irreversible once binding takes place. Binding appears to occur within the technical limits of the assay (ca. 1 min).

The heterodimer Mojave toxin (highly homologous to crotoxin) or just the basic subunit inhibit Ch uptake in the presence of BSA, however, the acidic subunit is ineffective in the absence or presence of BSA (Table 1). The incubation conditions included a 1 hr incubation at 25°C, which may be important with regard to the results with ACh release.

The effects of the *Naja naja atra* PLA<sub>2</sub>,  $\beta$ -Butx and scutoxin (100 nM) on Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent choline uptake were examined (Table 2). The inhibition of both Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent choline uptake was completely antagonized by BSA for the *Naja naja atra* PLA<sub>2</sub> and partially antagonized for scutoxin. There was no effect of BSA on the inhibition of Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent choline uptake by  $\beta$ -Butx. Under the conditions examined the Na<sup>+</sup>-independent uptake comprises about 35% of the total uptake in Na<sup>+</sup>-containing medium (data not shown). The Na<sup>+</sup>-independent choline uptake process is, if anything, less affected than total choline uptake (Table 2). Therefore, the toxins inhibit both choline uptake processes.

We have not had any success (despite considerable effort) in demonstrating any CONSISTENT effect of  $\beta$ -Butx, scutoxin, the *Naja naja atra* PLA<sub>2</sub>, or pseudexin on choline uptake in differentiated or undifferentiated PC12 or NB41A3 cells (numerous experiments - data not shown). We abandoned the use of these cell lines for studies of Ch uptake, especially since we have had considerable success with the mouse brain synaptosomes.

In conclusion, the PSNTXs irreversibly inhibit Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent choline uptake into synaptosomes, an action associated with the basic subunit for heterodimers, and this action is *not* completely inhibited by BSA (although some antagonism may occur). In contrast, inhibition of choline uptake by nonPSNTX PLA<sub>2</sub>s is completely inhibited by BSA.

### Acetylcholine Release

The dose-response relationships were examined as regards stimulation of ACh release. Numerous problems with the TLC-based analysis of ACh and Ch originally proposed for use

in the contract have caused us to use a previously described (Fletcher and Middlebrook, 1986) tetraphenyl- boron/3-heptanone-based extraction technique. Six toxins were examined at the time of the Mid-Term Report. The specific conditions examined were incubation with toxins at 37°C for 30 min. We found that BSA completely antagonizes the stimulation of ACh release by the *Naja naja atra* PLA<sub>2</sub>, presumably by removing the products of PLA<sub>2</sub> activity accessible to BSA at the outer leaflet of the membrane bilayer (see Figure 5A in Mid-Term Report). However, albumin has no effect, or even enhances the stimulatory effect of three of the PSNTXs ( $\beta$ -Butx, scutoxin, pseudexin) on ACh release (see Figures 5B, 5E and 5F, respectively, in Mid-Term Report). Since the PLA<sub>2</sub> activity of these three toxins was unaffected by the BSA treatment (see below), this suggests that these toxins have an action on ACh release unrelated to gross PLA<sub>2</sub> activity and that could relate to site-directed (intracellular leaflet of plasma membrane bilayer) PLA<sub>2</sub> activity, or an action independent of PLA<sub>2</sub> activity altogether. Two PSNTXs (taipoxin and crotoxin) had very little effect on ACh release (110% and 115% of control, respectively) in the presence of albumin when the studies were conducted at 37°C, even at 100 nM concentrations (see Figures 5C and 5D of Mid-Term report). However, this could relate to the specific incubation conditions and these toxins are discussed separately below.

Since the GC FA analysis (see below) was conducted with synaptosomes incubated with toxin at 25°C for 1 hr, we examined ACh release under these conditions. We found that BSA completely antagonizes the stimulation of ACh release by the *Naja naja atra* PLA<sub>2</sub> (Figure 3), as observed following incubation at 37°C for 30 min. In contrast to the results with the nonPSNTX PLA<sub>2</sub> (*N.n. atra* PLA<sub>2</sub>), albumin does not completely antagonize the stimulatory effect of three PSNTXs ( $\beta$ -Butx, pseudexin and scutoxin) on ACh release (Figure 3B, 3C and 3D). These studies are also in basic agreement with our previous findings at 37°C and 30 min.

Incubating the synaptosomes with the toxins for a brief period (1 min), washing the preparation free of unbound toxin and continuing the incubation for a total of 60 min did not decrease the percentage of ACh release stimulated by  $\beta$ -Butx or scutoxin compared to a 60 min incubation in which the toxin was present in the bath the entire time (Figure 4; Table 3). Therefore, most of the effects of the toxins on ACh release are irreversible once binding takes place. Binding appears to occur within the technical limits of the assay (ca. 1 min), in agreement with the choline uptake studies.

We tested an additional nonpresynaptically-acting PLA<sub>2</sub> (from *Naja naja kaouthia* venom) to verify that stimulation of ACh release would be antagonized by BSA, as with the *Naja naja atra* PLA<sub>2</sub> (Figure 5). The *Naja naja kaouthia* PLA<sub>2</sub> stimulates ACh release in the absence of BSA, but not in the presence of BSA. The effects on ACh release of the *Naja naja kaouthia* PLA<sub>2</sub> were directly compared to the *Naja naja atra* PLA<sub>2</sub> in the presence of BSA (Figures 6, 7 and 8). Since the *Naja naja kaouthia* PLA<sub>2</sub> is not a neurotoxic PLA<sub>2</sub>, this further suggests that our model system is capable of distinguishing between neurotoxic and nonneurotoxic PLA<sub>2</sub>s by the absence of a stimulatory effect on ACh release in the presence of BSA.

When BSA (0.5%) is included in the incubation medium, the effects of  $\beta$ -Butx on ACh release become apparent at 5 min, in contrast to the minimal effect of the toxin at that time in the absence of BSA (Figure 6). Increasing the concentration of BSA to 1% may somewhat

increase the effects of the neurotoxins (compare Figure 6 to Figure 7). Since BSA removes fatty acids we had anticipated an inhibitory effect of increasing the BSA concentration, if anything. In Figure 7 it becomes apparent that a short-term (5 min) incubation with toxin enhances ACh release relative to controls to a greater extent than a longer term (1 hr) incubation (Figure 7). This trend of a short-term incubation with toxin resulting in a greater relative increase in ACh release than a long-term incubation (in the presence of BSA) is confirmed with a slightly different approach in Figure 9 (30 vs. 60 min; add neostigmine for 4 min) and by the same approach as in Figures 6 and 7 in Figure 8 (15, 30, 60 min). Therefore, in the presence of BSA, the toxin effects have a rapid onset that becomes less obvious as time progresses. Including neostigmine in our highly purified synaptosomal preparation has very little beneficial effect (Figure 10). In a second study, using the same conditions (60 min toxin exposure; 4 min neostigmine) as in Figure 10,  $\beta$ -Butx 100 nM in the absence of neostigmine induced [mean  $\pm$  SD (n)]  $113 \pm 2.9\%$  (3) of control ACh release and in the presence of neostigmine 100  $\mu$ M induced  $110 \pm 1.5\%$  (3) control.

We examined the effects of the PSNTXs on ACh release from rat brain synaptosomes and have found the results to be somewhat comparable to mouse brain synaptosomes, as regards nonPSNTXs,  $\beta$ -Butx, scutoxin and pseudexin. In the presence of BSA (0.5%)  $\beta$ -Butx, scutoxin and pseudexin at a 100 nM concentration and 60 min incubation time all stimulated ACh release (Figure 11), with pseudexin being the least efficacious at the 100 nM concentration. The *Naja naja kaouthia* PLA<sub>2</sub> was relatively inactive (Figure 11), as was previously observed with mouse brain preparations. Although we have occasionally observed greater effects on ACh release by pseudexin in mouse brain synaptosomes, we have frequently observed this similar low level of stimulation of ACh release (see below). The time course of toxin action in rat brain synaptosomes was examined using  $\beta$ -Butx (Figure 12). The stimulation of ACh release is apparent at the shortest time point examined (5 min) and appears to reach a maximum at 30 min.

In our earlier studies using mouse brain synaptosomes, two PSNTXs (taipoxin and crotoxin) had very little effect on ACh release (110% and 115% of control, respectively) in the presence of albumin, even at 100 nM concentrations (see Figures 5C and 5D of Mid-Term report). We have subsequently tested the effects on ACh release of taipoxin, crotoxin and mojave toxin in rat brain synaptosomes (Figures 13 and 14). In contrast to our findings in mouse brain synaptosomes in which mojave and crotoxin were inactive, these toxins were comparable to  $\beta$ -Butx in stimulation of ACh release (Figures 13 and 14). The "dose-response" (10 vs. 100 nM) for mojave toxin (Figure 14) appeared similar to that we previously observed with  $\beta$ -Butx in mouse brain synaptosomes. Overall, in three separate experiments, mojave toxin (100 nM) induced ACh release that was  $131 \pm 4.7\%$  of control (means  $\pm$  SEM; n = 3). Taipoxin was the least efficacious toxin of those tested (Figures 13 and 14). The dose-response for  $\beta$ -Butx was examined in the rat preparation (Figure 15). Therefore, the rat preparation appeared to be more sensitive to the toxin than the mouse preparation.

Other studies have been conducted with synaptosomes from mouse brain. Since the newer GC analysis of FA studies were conducted in the presence of BSA at a concentration of 1% (we formerly used 0.5%), we contrasted the effects of the toxins on ACh release at the two BSA concentrations (Figure 16). Increasing the BSA concentration from 0.5 to 1%

significantly increased ACh release induced by the *Naja naja atra* and *Naja naja kaouthia* PLA<sub>2</sub>s and  $\beta$ -Butx, but did not have a significant effect on ACh release induced by scutoxin or pseudexin (Figure 16).

We have examined the effects of monoclonal antibodies developed to a mixture of pseudexins A, B and C (Middlebrook, 1991) on the enzymatic and ACh releasing activities of the presynaptically-acting neurotoxins. Two monoclonal antibodies (#1 and #3) provided by Dr. John Middlebrook (USAMRIID, Ft. Detrick, Frederick, MD) were first examined to verify their effects on the enzymatic activity of pseudexin on artificial substrates (Figure 17). In agreement with previously published data (Middlebrook, 1991), monoclonal #3, but not #1, antagonized the enzymatic activity of pseudexin B, as determined by titration with egg yolk phosphatidylcholine and Triton X-100 (1:2). Also, monoclonal antibody #3 decreased ACh release by pseudexin B (Figure 18). Antibody #3 cross reacts with notexin (one amino acid different from scutoxin) by Elisa (Middlebrook, 1991) and also inhibits ACh release by scutoxin in the absence or presence of BSA (Figure 19). The antibodies in the absence of toxin tend to increase ACh release from mouse brain synaptosomes in all cases (Figure 20). Antibody #2 was the least active in stimulating ACh release. Of the antibodies examined in the present study, only #3 and #7 have been reported to be neutralizing to pseudexin (Middlebrook, 1991). These two antibodies also were reported to inhibit PLA<sub>2</sub> activity (Middlebrook, 1991). In agreement with these previous reports only antibodies #3 and #7 inhibited ACh release by pseudexin (Figure 21). Since the antibodies to pseudexin exhibit varying degrees of cross-reactivity to other PSNTXs by ELISA (Middlebrook, 1991), we examined the effects of the antibodies on scutoxin and  $\beta$ -Butx-induced ACh release. Notexin (an analogue of scutoxin) was recognized to the same extent as pseudexin by monoclonal antibodies #3 and #4, almost to the same extent by antibodies #2 and #7 and to a considerably lesser extent by antibodies #1 and #12 (Middlebrook, 1991). In agreement with the above, the two cross-reacting and neutralizing antibodies (#3 and #7) were the only two to antagonize scutoxin-induced ACh release (Figure 22).  $\beta$ -Butx was overall less cross-reactive to the antibodies (except for #2 and #4) than notexin (Middlebrook, 1991).  $\beta$ -Butx was not cross-reactive with #7 at all and was about 10-fold less reactive than notexin to #3 (Middlebrook, 1991). None of the antibodies directed toward pseudexins inhibited  $\beta$ -Butx-induced ACh release (Figure 22).

One concern we have had is whether the release of radioactive ACh reflects the release of total ACh. This concern arises since the pools of radiolabeled ACh may not reflect the normally released pools of ACh. Therefore, in collaboration with Dr. Robert Storella (Hahnemann University, PA), we have examined the effects of the *Naja naja atra* PLA<sub>2</sub>,  $\beta$ -Butx and pseudexin on total ACh release from mouse brain synaptosomes as determined by HPLC analysis (electrochemical detection). The results with total ACh release (HPLC) (Table 4) are in reasonable agreement with the results using radiolabeled ACh (see Figure 5 in Mid-Term Report).

As with the Ch uptake studies, we have not had any success (despite considerable effort) in demonstrating any CONSISTENT effect of  $\beta$ -Butx, scutoxin, the *Naja naja atra* PLA<sub>2</sub>, or pseudexin on ACh release in differentiated or undifferentiated PC12 or NB41A3 cells (numerous experiments - data not shown). We abandoned the use of these cell lines for studies of ACh release, especially since we had considerable success with the mouse brain

synaptosomes.

We examined a synthetic homologue of a toxin from *Trimeresurus wagleri* snake venom (Weinstein et al., 1991) using the phrenic nerve-diaphragm preparation. We noticed that pretreatment with succinylcholine (50  $\mu$ M) unmasked a specific presynaptic effect of the toxin (see Table 1 in Mid-Term Report).

In conclusion, the PSNTXs irreversibly stimulate ACh release from mouse and rat brain synaptosomes and this action is *not* completely inhibited by BSA (although some antagonism may occur). This action has an early onset (ca. 5 min) and diminished with time (ca. >15 min). The stimulation of ACh release by crotoxin, moiave toxin and taipoxin appears to be much more obvious in rat than in mouse brain synaptosomes. Increasing the BSA concentration from 0.5 to 1.0% increases the efficacy of the toxins in stimulating ACh release. Two antibodies to pseudexins A, B and C, effective in neutralizing pseudexin *in vivo* (LD<sub>50</sub>), also antagonized the stimulation of ACh release by pseudexin and scutoxin, but not  $\beta$ -Butx. Nonneutralizing antibodies had no effect on ACh release. In contrast to PSNTXs, nonPSNTX PLA<sub>2</sub>s did not stimulate ACh release in the presence of BSA. A nonPLA<sub>2</sub> peptide from *Trimeresurus wagleri* snake venom is also a PSNTX.

#### Effects of PSNTXs on Lipid Metabolism in Nerve and Muscle Cell Culture Systems

The studies of PSNTXs in human skeletal muscle cultures were conducted to test the effects of these toxins on postsynaptic lipid metabolism and to set the stage for future studies with nerve cells. Exposure of primary cultures of human skeletal muscle to  $\beta$ -Butx for 2 hrs did not cause high levels of release of radioactivity to the incubation medium (< 3%) similar to those observed with CTX and melittin. Since linoleic acid primarily labels the #2 position of phospholipids, the release of FFAs is the most sensitive indicator of PLA<sub>2</sub> activity. However, since some radiolabel is attached to the #1 position of phospholipids, lysophosphatidylcholine production can be followed at higher levels of PLA<sub>2</sub> activity. In the absence of BSA, the level of FFA was slightly (about 3% of the total lipid associated radiolabel), but significantly ( $P < 0.01$ ; two-tailed t-test) raised at a concentration of  $\beta$ -bungarotoxin of 0.1  $\mu$ M (see Figure 8 in Mid-Term Report). The levels of PLA<sub>2</sub> activity as determined by the formation of FFAs and lysophospholipids were considerably greater at higher concentrations of toxin (see Figure 8 in Mid-Term Report). There was no change in the levels of diacylglyceride or triglyceride upon treatment with the toxin, suggesting that the PSNTXs do not activate tissue PLC or TG lipase, nor do they possess these actions.

We have also examined the phospholipid hydrolysis induced by the PSNTXs in two nerve cell lines (PC12, NB41A3). Even at a concentration of 100 nM and with BSA in the incubation medium, the PSNTXs do not release radioactivity into the medium (< 3%; data not shown) to the same extent as CTX and melittin (see below). Phospholipid hydrolysis by PLA<sub>2</sub>s in our system is best evidenced by increases in FFAs (indicates overall activity - fatty acids are a direct product from PLA<sub>2</sub> hydrolysis of any substrate), increases in LPC (a direct product of PC hydrolysis) and decreases in PE or PS (a decrease is due to greater hydrolysis of PE or PS relative to the other phospholipids). There appears to be considerable variability in the extent to which the phospholipids are hydrolyzed by any one of the enzymes and this relates to the time of incubation, the presence of BSA (which greatly enhances PLA<sub>2</sub> activity) and other yet-to-be-identified factors. Hydrolysis induced by  $\beta$ -Butx is low relative to the

*Naja naja atra* and *Naja naja kaouthia* PLA<sub>2</sub>s, scutoxin and pseudexin, although at times it can be similar to the other PLA<sub>2</sub>s (note the large SD values for  $\beta$ -Butx in Table 5). The hydrolysis of PE is particularly low for  $\beta$ -Butx, although this is not a general characteristic of PSNTXs (Table 5). There is a feature of phospholipid hydrolysis common to the PSNTXs. PS is not an equivalent or preferred substrate relative to PE for the two nonneurotoxic (*Naja naja atra*, *Naja naja kaouthia*) PLA<sub>2</sub>s and this is not observed for the three PSNTXs tested (Table 5). These findings support a potential site-directed PLA<sub>2</sub> activity.

We have also extended the studies of phospholipid hydrolysis induced by the PSNTXs in one of the nerve cell lines (NB41A3) by examining lower concentrations (10 nM) of toxin. The cells were radiolabeled with either unsaturated (18:2) or saturated (18:0) fatty acids. A major problem encountered in our studies at 100 nM toxin concentrations that we have now overcome through the employment of a ten-fold lower concentration was the extensive phospholipid hydrolysis induced by the PSNTXs in the presence of BSA. This high level of hydrolysis masked some effects that were much more evident at lower toxin concentrations.

#### ***Studies in Differentiated Cells***

***Studies radiolabeling cells with 18:2.*** As judged by free fatty acid release, total phospholipid hydrolysis induced by 10 nM concentrations of  $\beta$ -Butx and scutoxin are low relative to the *Naja naja atra* and *Naja naja kaouthia* PLA<sub>2</sub>s and pseudexin (Figure 23A). The hydrolysis of PC (judged by LPC production) was also low for  $\beta$ -Butx and scutoxin relative to the *Naja naja kaouthia* PLA<sub>2</sub> and pseudexin (Figure 23A). The *Naja naja atra* PLA<sub>2</sub> also had a low substrate preference for PC (Figure 23A). The hydrolysis of PE is particularly low for all three PSNTXs and yet this phospholipid is readily hydrolyzed by the nonPSNTXs (Figure 23B). PS appears to be somewhat resistant to hydrolysis by the two nonPSNTXs (Figure 23B). Therefore, PE is a preferred substrate relative to PS for the two nonneurotoxic (*Naja naja atra*, *Naja naja kaouthia*) PLA<sub>2</sub>s. A similar conclusion was reached on the hydrolysis of purified substrates by two nonPSNTX PLA<sub>2</sub>s (*Naja naja atra* and *Hamachatus haemachatus*; Condrea et al., 1981). Such a substrate preference of PE over PS has not been observed for the three PSNTXs at the 10 nM (Figure 23B) or 100 nM (Table 5) concentrations. These findings support a potential PS-associated site-directed substrate specific PLA<sub>2</sub> activity specific to the PSNTXs.

***Studies radiolabeling cells with 18:0.*** Only the *Naja naja kaouthia* PLA<sub>2</sub> and pseudexin were significantly active on substrate radiolabeled with 18:0 (Figure 24A). While both enzymatic activities were high toward PC (Figure 24B), pseudexin had a greater preference for PS than did the *Naja naja kaouthia* PLA<sub>2</sub> (Figure 24C). The *Naja naja kaouthia* enzyme had a greater preference for PE than did pseudexin (Figure 24D) in agreement with our finding using 18:2 labeling.

#### ***Studies in Nondifferentiated Cells***

The findings in nondifferentiated NB41A3 cells radiolabeled with 18:2 were similar to those in differentiated cells radiolabeled with 18:0. That is, only hydrolysis by the *Naja naja kaouthia* PLA<sub>2</sub> and pseudexin was significant. (Figure 25A). While the *Naja naja kaouthia* PLA<sub>2</sub> readily hydrolyzed PC (Figure 25B) and PE (Figure 25D), the activity toward PS was minimal (Figure 25C). In contrast, pseudexin appeared to equally hydrolyze all three major phospholipids (Figure 25A-D).

### ***Studies of BSA Effects on Phospholipase A<sub>2</sub> Activity***

Since BSA antagonizes specifically the actions of the two PSNTXs on ACh release, the effects of BSA on PLA<sub>2</sub> activity in the NB41A3 cell line was examined in a separate set of studies. Total PLA<sub>2</sub> activity (reflected best by the total FFA release) was increased for both nonPSNTX PLA<sub>2</sub>s and for pseudexin (Figure 26A). The trends toward an increase in activity for  $\beta$ -Butx and scutoxin were not significant. Similarly, the hydrolysis of PC (reflected by an increase in LPC) was only increased for the two nonPSNTX PLA<sub>2</sub>s and for pseudexin (Figure 26B). The hydrolysis of PS is specifically decreased by BSA addition (i.e., levels of PS remain higher), but only for the two nonPSNTXs (Figure 26C). The levels of PE are significantly decreased for all conditions, including controls in the presence of BSA (Figure 26D). Quantitatively, phospholipid hydrolysis, especially of PE, was increased by BSA. The main qualitative effect of BSA appeared to be to increase hydrolysis of PE relative to PS by the two nonPSNTXs (Figure 26D).

### ***Effects of PSNTXs on Fatty Acid Production in Synaptosomes***

These studies were designed to determine whether BSA truly extracts the FFAs from the synaptosomal preparations and whether the more toxic PLA<sub>2</sub> neurotoxins produce FFAs that are inaccessible to BSA, suggesting that either they are produced in the inner layer of the membrane bilayer, or they result from a difference between PSNTX and nonPSNTX in substrate specificity. In brief, the synaptosomes are incubated  $\pm$  BSA and with either *Naja naja atra* PLA<sub>2</sub>, a PSNTX or no toxin. The synaptosomes are pelleted by centrifugation and the supernatant ( $\pm$  BSA) is removed. The supernatant and pellet are then extracted and the FFAs analyzed. In the absence of BSA, the release of free fatty acids into the incubation medium (supernatant following centrifugation of synaptosomal suspension) by any of the toxins (Figures 27A and 29A) was minimal relative to the fatty acid generation in the synaptosomes (pellet following centrifugation of synaptosomal suspension; Figures 27B and 29B). The values for total free fatty acids in the control synaptosomal preparations (Figures 27B and 29B) were in agreement with those of other investigators using a similar synaptosomal preparation (Rhoads et al., 1983). There was very little hydrolysis of 16:1, 18:0 and 18:2 by any of the PLA<sub>2</sub>s (Figures 27 and 29). The most abundant fatty ester at the #2 position in synaptosomal phospholipids, as determined by PLA<sub>2</sub> hydrolysis, was 22:6, followed by about equal amounts of 16:0, 18:1 and 20:4.

With some minor exceptions, addition of BSA to the Incubation Medium removed a similar amount of fatty acid generated by the PLA<sub>2</sub>s from the synaptosomes (pellet following centrifugation) into the Incubation Medium (supernatant following centrifugation) for all toxin-treated preparations (Figures 28A and 30A). The patterns of fatty acids removed by BSA were also similar for all of the PLA<sub>2</sub>s, suggesting that these fatty acids are derived from the same pool, presumably the outer leaflet of the membrane bilayer, and that this pool is available to BSA regardless the PLA<sub>2</sub> employed. A much greater proportion of 20:4 relative to 16:0 and 18:1 was removed from the synaptosomal membranes into the Incubation Medium by BSA (Figures 28A and 30A) than remained in the synaptosomes in either the absence (Figures 27B and 29B) or presence (Figures 28B and 30B) of BSA.

The values for total free fatty acids in the control synaptosomal preparations treated with BSA (Figures 28B and 30B) were also in agreement with those of other investigators using



BSA to remove fatty acids from synaptosomal preparations (Rhoads et al., 1983). The three phospholipid fatty esters, 16:1, 18:0 and 18:2, were also not hydrolyzed to any extent by the toxins in the presence of BSA in the Incubation Medium (Figures 28 and 30), in agreement with the virtual absence of hydrolysis in the absence of BSA (Figures 27 and 29). The amount of 16:0 and 18:1 that was retained in the synaptosomes in a BSA-containing medium by  $\beta$ -bungarotoxin, scutoxin and pseudexin was much greater than observed with the *Naja naja atra* PLA<sub>2</sub>. Since BSA was unable to remove these two fatty acids, this would suggest that they were not produced by PLA<sub>2</sub> activity in the outer leaflet of the plasma membrane bilayer. These fatty acids (16:0 and 18:1) were the only two consistently resistant to removal by BSA from the synaptosomes (relative to those produced by the *Naja naja atra* PLA<sub>2</sub>) for the 10 and 100 nM concentrations of all three of the PLA<sub>2</sub> neurotoxins. At these concentrations the neurotoxins, but not the *Naja naja atra* PLA<sub>2</sub>, stimulate ACh release and  $\beta$ -bungarotoxin and scutoxin antagonized choline uptake. Pseudexin B at a 10 nM concentration did not significantly antagonize choline uptake and was the least effective neurotoxin in stimulating ACh release. Pseudexin B at the 10 nM concentration also produced less 16:0 that was retained in the synaptosomes in the presence of BSA than did the the two other neurotoxins (Figure 28B), providing further evidence that this fatty acid may be important in modulating choline uptake and ACh release.

The total PLA<sub>2</sub> activities in Figure 30 (the supernatant Panel A plus the pellet in Panel B) were compared to the PLA<sub>2</sub> activities in Figure 29 to examine whether BSA affected enzyme activity. At the 100 nM concentration of toxins and in the absence of BSA,  $\beta$ -bungarotoxin ( $75 \pm 7$  nmol/mg synaptosomal protein) and the *Naja naja atra* PLA<sub>2</sub> ( $77 \pm 14$  nmol/mg protein) produced similar levels of total (supernatant plus pellet) free fatty acids, while scutoxin ( $130 \pm 14$  nmol/mg protein) and pseudexin ( $147 \pm 13$  nmol/mg protein) produced higher levels. In the presence of BSA (0.5%) there were significance differences between the presynaptically-acting neurotoxins ( $\beta$ -bungarotoxin, pseudexin and scutoxin) and the *Naja naja atra* PLA<sub>2</sub> in total free fatty acids, 16:0 and 18:1 (Figure 30). Addition of BSA to the bathing medium significantly ( $P < 0.01$ ; two-tailed t-test) increased total (pellet plus supernatant) free fatty acid production (to  $106 \pm 13$  nmol/mg protein) by  $\beta$ -bungarotoxin. Free fatty acid production was not enhanced by BSA for the *Naja naja atra* PLA<sub>2</sub> ( $75 \pm 3$  nmol/mg protein), pseudexin ( $158 \pm 11$  nmol/mg protein) or scutoxin ( $131 \pm 7$  nmol/mg protein).

We have also analyzed the fatty acids liberated in synaptosomal preparations by the neurotoxic and nonneurotoxic PLA<sub>2</sub>s incubated in a 1% BSA-containing buffer. The total PLA<sub>2</sub> activity (overall production of free fatty acids) had no relationship to stimulation of ACh release, as fatty acid production by  $\beta$ -Butx did not differ from the two nonPSNTX PLA<sub>2</sub>s (Figure 31A). This was evident whether total fatty acid production, or just the fatty acids retained in synaptosomal membranes or extracted by BSA were examined (Figure 31A). Likewise, the liberation of total unsaturated fatty acids (18:1; 20:4; 22:6) had no relationship to stimulation of ACh release. The lack of relationship of hydrolysis of unsaturated fatty acids to presynaptic neurotoxicity was true whether total, 20:4 (Figure 31B), 18:1, or 22:6 (Figure 31C) were examined. In contrast to the liberation of unsaturated fatty acids, the total (pellet plus supernatant) saturated fatty acids produced was greater for the three PSNTXs examined than for the two nonPSNTX PLA<sub>2</sub>s (Figure 31D). This relationship was not as evident when

the saturated fatty acids were subclassified into the total retained in the synaptosomes (pellet; Figure 31D), or into the individual 16:0 and 18:0 produced in the pellet or supernatant (Figure 31E). However, it was clear that the PSNTX PLA<sub>2</sub>s overall had a greater substrate specificity for phospholipids containing saturated fatty acids at the #2 position.

In conclusion, the PSNTXs do not activate tissue lipases in cell cultures. The PSNTXs are distinguished from the nonPSNTX PLA<sub>2</sub>s in that the PSNTXs hydrolyze PS to the same extent as PE; whereas PS is not as good a substrate as PE for the nonPSNTX PLA<sub>2</sub>s. The PSNTXs more readily hydrolyze differentiated over nondifferentiate nerve cells and less readily hydrolyze muscle culture phospholipids. In cell cultures the addition of BSA increases or has no effect on phospholipid hydrolysis; however, the substrate preference of the nonPSNTX PLA<sub>2</sub>s increases for PE. In synaptosomes, there was no correlation between total FAs generated and presynaptic activity of the PSNTXs or non PSNTX PLA<sub>2</sub>s. At a concentration of BSA of 0.5%, there was a consistent resistance to extraction from the synaptosomes of 16:0 and 18:1 produced by PSNTXs, suggesting that the PSNTXs were reaching a site inaccessible to the nonPSNTX PLA<sub>2</sub>s and BSA. Increasing the BSA concentration to 1% revealed a greater overall hydrolysis of unsaturated FAs (16:0; 18:0) by the PSNTXs. Therefore, the PSNTXs have a very different spectrum of phospholipid hydrolysis from the nonPSNTX PLA<sub>2</sub>s.

#### Effects of a PSNTX ( $\beta$ -Butx) on the Threshold of $\text{Ca}^{2+}$ -induced $\text{Ca}^{2+}$ Release (TCICR)

We have also examined the effects of  $\beta$ -Butx on  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum. In agreement with other investigators (Lau et al., 1974), we have observed that  $\beta$ -Butx causes  $\text{Ca}^{2+}$  release from terminal cisternae preparations (Figure 32). Like melittin, CTX and myotoxin a,  $\beta$ -Butx decreases the TCICR in terminal cisternae preparations from skeletal muscle (Figure 32). Addition of  $\beta$ -Butx after preloading the HSRFs (see Fletcher et al., 1991b) results in an immediate release of  $\text{Ca}^{2+}$  that is blocked by ruthenium red (Figure 32).  $\beta$ -Butx is similar to CTX in its mechanism of  $\text{Ca}^{2+}$  release, as sufficient  $\text{Ca}^{2+}$  preload is required for the action of  $\beta$ -Butx (Figure 32), as is required for CTX (Fletcher et al., 1993).

In conclusion,  $\beta$ -Butx decreases the TCICR from intracellular  $\text{Ca}^{2+}$  stores through an action at the ryanodine receptor.

### **Results - CTXs, Melittin and Myotoxins**

#### Effects of CTXs, Melittin and Myotoxin a on Calcium Regulation

#### Effects of CTXs, melittin and myotoxin a on the Threshold of $\text{Ca}^{2+}$ -induced $\text{Ca}^{2+}$ Release (TCICR)

The CTX from *Naja naja kaouthia* venom was previously reported to decrease the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in porcine terminal cisternae preparations when injected after ATP-stimulated  $\text{Ca}^{2+}$  uptake had reached equilibrium (Fletcher et al., 1991b). However, the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release was not decreased by CTX in equine preparations using the same paradigm (Fletcher et al., 1993). The threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in human preparations was more sensitive ( $P < 0.0005$ ; two-tailed grouped t-test) to CTX than that in equine preparations (Fletcher et al., 1993). A 100-fold lower concentration of melittin ( $0.1 \mu\text{M}$ ) was as efficacious as a  $10 \mu\text{M}$  concentration of CTX in reducing the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from human preparations (Fletcher et al., 1992). Higher

concentrations of melittin ( $1\ \mu\text{M}$ ) immediately released extremely large amounts of  $\text{Ca}^{2+}$  (beyond the upper limits of the arsenazo III range) and this appeared to be due to lysis of the vesicles (Fletcher et al., 1992). Unlike the  $0.1\ \mu\text{M}$  concentration of melittin, the release of  $\text{Ca}^{2+}$  by these higher concentrations did not require prior  $\text{Ca}^{2+}$  additions (Fletcher et al., 1992). In contrast to CTX, there was no significant difference ( $P > 0.05$ ) between the effects of melittin on human and equine muscle (Fletcher et al., 1992). As indicated by the large standard error of the mean and the wide range of values, there was a large variation in the effects of CTX (Fletcher et al., 1993) or melittin (Fletcher et al., 1992) on the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, depending on the individual horse or human examined. Since the species difference in the action of CTX was unexpected, we verified that CTX ( $10\ \mu\text{M}$ ) induced contractures in equine muscle fiber bundles (Fletcher et al., 1993) and found that these contractures were similar in all respects to those in a  $\text{Ca}^{2+}$ -containing medium previously reported for human fiber bundles (Fletcher and Lizzo, 1987).

To test whether CTX had *any* action on  $\text{Ca}^{2+}$  release from equine muscle, terminal cisternae preparations were preloaded to 29-92% of the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release and were then challenged with CTX (Fletcher et al., 1993). Eleven of the 14 equine preparations preloaded to greater than 65% of the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release immediately released  $\text{Ca}^{2+}$  upon subsequent CTX addition (Fletcher et al., 1993). Of the 6 equine preparations preloaded to 65% or less of the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, none exhibited any indication of  $\text{Ca}^{2+}$  release in response to CTX (Fletcher et al., 1993). Similar results were obtained with human preparations. All eight human preparations preloaded to greater than 65% of the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release exhibited  $\text{Ca}^{2+}$  release when challenged with CTX (Fletcher et al., 1993). One of four human preparations preloaded to 65% or less of the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release exhibited  $\text{Ca}^{2+}$  release upon challenge with CTX (Fletcher et al., 1993).

Even trace contamination of the CTX fraction with the *Naja naja kaouthia* snake venom  $\text{PLA}_2$  has been suggested to account for many of the actions of CTX (Fletcher et al., 1991a). Therefore, the CTX fraction was pretreated with *p*-BPB to considerably reduce the contribution of venom  $\text{PLA}_2$  to the CTX action. This treatment had no effect on CTX reduction of the TCICR (Fletcher et al., 1991a), indicating that trace contamination with venom  $\text{PLA}_2$  activity is not involved in the rapid activation of  $\text{Ca}^{2+}$  release by the toxin.

Synthetic melittin was used to avoid similar  $\text{PLA}_2$  problems associated with venom-derived melittin (Fletcher et al., 1990a). We verified that synthetic melittin induced contractures in equine skeletal muscle (Fletcher et al., 1992), equine; unpublished observations), as previously reported for venom-derived melittin (Lin Shiau et al., 1975). Melittin also elicited  $\text{Ca}^{2+}$  release from  $\text{Ca}^{2+}$  preloaded terminal cisternae-containing fractions from equine and human muscle (Fletcher et al., 1992). As observed with CTX, melittin did not induce  $\text{Ca}^{2+}$  release if the terminal cisternae preparations were insufficiently preloaded with  $\text{Ca}^{2+}$  (Fletcher et al., 1992). In some preparations melittin caused a slight, but transient, release of  $\text{Ca}^{2+}$  at low  $\text{Ca}^{2+}$  preloads (Fletcher et al., 1992).

We confirmed that in the absence of CTX the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, as determined in the present study, is decreased in equine and human muscle by ryanodine ( $30\ \mu\text{M}$ ), which opens the  $\text{Ca}^{2+}$  release channel, and this action is antagonized by ruthenium red ( $10\ \mu\text{M}$ ) (Fletcher et al., 1993), as reported for rabbit muscle by other investigators using

similar methodology (Palade, 1987b; Zimanyi and Pessah, 1991). The action of CTX in human preparations was antagonized by ruthenium red (10  $\mu$ M) whether determined by the effects on the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, or on preloaded preparations (Fletcher et al., 1993). Melittin-induced  $\text{Ca}^{2+}$  release from human and equine preparations was antagonized by ruthenium red (Fletcher et al., 1992). A slight transient release of  $\text{Ca}^{2+}$  by melittin was observed in some preparations even in the presence of ruthenium red.

Since dantrolene partially antagonized contractures to CTX in rat and human skeletal muscle (Fletcher and Lizzo, 1987), we examined the effects of dantrolene on CTX action in terminal cisternae preparations. Dantrolene (10  $\mu$ M) did not antagonize  $\text{Ca}^{2+}$  release by CTX in either equine or human preparations (Fletcher et al., 1993).

Like melittin and CTX, myotoxin *a* also decreases the threshold of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (TCICR) in terminal cisternae preparations from skeletal muscle (Yudkowsky et al., 1994). Unlike CTX, but rather similar to melittin, myotoxin *a* is about equipotent on equine and human skeletal muscle (Yudkowsky et al., 1994).  $\text{Ca}^{2+}$  release by myotoxin *a* (10  $\mu$ M) is blocked by ruthenium red (10  $\mu$ M; Yudkowsky et al., 1994) and is unaffected by verapamil (1  $\mu$ M; see Table 13 in Mid-Term Report). Addition of myotoxin *a* after preloading the HSRFs results in an immediate release of  $\text{Ca}^{2+}$  that is blocked by ruthenium red (Yudkowsky et al., 1994). We tested the effects of myotoxin *a* on the  $\text{Ca}^{2+}$  pump. Triplicate determinations of  $\text{Ca}^{2+}$  uptake were determined for each condition. In the absence of ruthenium red, myotoxin *a* (10  $\mu$ M) inhibited  $\text{Ca}^{2+}$  uptake (Table 6). Ruthenium red, a blocker of  $\text{Ca}^{2+}$  release, abolished the "apparent" inhibition of ATP-stimulated  $\text{Ca}^{2+}$  uptake by myotoxin *a*; that is, when true uptake was determined (Table 6). These findings do not support inhibition of the  $\text{Ca}^{2+}$  pump by myotoxin *a* as causing  $\text{Ca}^{2+}$  release, but rather the opening of the  $\text{Ca}^{2+}$  release channel being the primary mode of action.

#### ***Effects of CTX, Melittin and Myotoxin a on Ryanodine Binding to the $\text{Ca}^{2+}$ Release Channel***

Melittin and cardiotoxin were found to have dual effects on the binding of ryanodine to the  $\text{Ca}^{2+}$  release channel (ryanodine receptor) of skeletal muscle and these effects were dependent on the preparation of sarcoplasmic reticulum employed. In one case (see Figure 14A of Mid-Term Report) melittin at a low concentration (0.01  $\mu$ M) and CTX at a higher concentration (10  $\mu$ M) inhibited [ $^3\text{H}$ ]ryanodine binding. In this case a terminal cisternae-containing fraction (4,000 - 12,000  $\times$  g) was isolated and this fraction did not contain significant amounts of cytosolic protein or light membrane vesicles. A fraction containing these cellular components (see Figure 14B of Mid-Term Report) manifests an enhancing action of melittin and CTX on ryanodine binding. However, at the concentrations used to induce  $\text{Ca}^{2+}$  release (0.1  $\mu$ M for melittin and 10  $\mu$ M for CTX) neither toxin had significant effects on ryanodine binding under either condition.

Myotoxin *a* also altered ryanodine binding to the calcium release channel in skeletal muscle (Yudkowsky et al., 1994). The calcium release channel exists in at least two functional states and these can be differentiated by the  $K_d$  of ryanodine binding (Hawkes et al., 1992). Myotoxin *a* converts the channel from a low-affinity state to a higher affinity state (Yudkowsky et al., 1994). In those animals in which the calcium release channel is already in a high-affinity state, there is no further effect of myotoxin *a* (Yudkowsky et al., 1994).

In conclusion, the CTX from *Naja naja kaouthia* venom, melittin and myotoxin *a* all

induce  $\text{Ca}^{2+}$  release from terminal cisternae preparations and this action is mediated through the ryanodine receptor ( $\text{Ca}^{2+}$  release channel). There is more species variability in the action of the CTX than melittin or myotoxin *a*. There is considerable interindividual variability within species for all three toxins. The effects on the channel appear to be very similar, yet slightly different for the three toxins, especially based on the ryanodine binding studies.

#### ***Effects of CTXs, Melittin and Myotoxins on Cellular Lipid Metabolism***

##### ***Effects of p-BPB Treatment on CTX- and Melittin-Induced Lipid Metabolism in Cell Culture Systems***

To examine if venom  $\text{PLA}_2$  contamination could play a major role in the effects of the CTX fractions on lipid metabolism in primary cultures, as was observed with venom-derived melittin (Fletcher et al., 1990a), we tested the relatively more highly contaminated CTX fraction from *N. n. atra* venom (Fletcher et al., 1991a). Treatment with this CTX exhibited extensive  $\text{PLA}_2$  activity that could be greatly reduced by treating the fraction with p-BPB prior to incubation with the cells. The patterns of hydrolysis by obvious  $\text{PLA}_2$  contamination in the native fraction (Fletcher et al., 1991a) do not match those produced by the less  $\text{PLA}_2$ -contaminated fraction from CTX from *N. n. kaouthia* venom (Fletcher et al., 1991a).

By greatly reducing  $\text{PLA}_2$  activity in the CTX fraction with p-BPB, we totally eliminate the production of FFAs by the *Naja naja kaouthia* CTX in a mouse cell line (see Figure 6A in Mid-Term Report). The native toxin causes significant production of LPC, which is associated with  $\text{PLA}_2$  activity (see Table 3 in Mid-Term Report). Interestingly, in the mouse cell line there was no significant increase in diacylglycerol, the major product of PLC activity (see Figure 6B in Mid-Term Report), suggesting that the PLC enzyme was not present in the cell line, at least at the time tested.

##### ***Effects of CTX, Melittin and Myotoxin a on Lipid Metabolism in Cell Culture***

About 4% of the [ $^{14}\text{C}$ ]linoleic acid radiolabel is incorporated into the cells three days after adding the FA to the cultures (Fletcher et al., 1991a). CTX action produces FFAs and diacylglycerol (Fletcher et al., 1991a). The effects of CTX appeared to be completely accounted for by hydrolysis of the phospholipid fraction. There was no obvious preference for hydrolysis of any one particular phospholipid by the CTXs.

The effects of melittin were examined in primary cultures of equine skeletal muscle (Fletcher et al., 1991a). At a low concentration (2  $\mu\text{M}$ ) the effects of melittin on lipid metabolism seemed similar to the higher (10  $\mu\text{M}$ ) concentration of CTX. That is, melittin increased the diacylglyceride and free fatty levels, as observed for CTX. However, at higher concentrations of melittin (10  $\mu\text{M}$ ) significant triglyceride breakdown was also evident (Fletcher et al., 1991a). Despite an increase in FA production by the higher concentration of melittin, the amount of radioactivity released into the medium was lower than that released by the 2  $\mu\text{M}$  concentration (Fletcher et al., 1991a).

The effects of CTX and melittin were examined in human muscle to determine if substantial species differences exist in regard to the toxin action (Fletcher et al., 1991a). CTX and melittin had similar actions in human and equine skeletal muscle cultures. While there was no significant difference between CTX (10  $\mu\text{M}$ ) and melittin (10  $\mu\text{M}$ ) in amount of radioactivity released from human muscle cells to the supernatant ( $P > .05$ ; two-tailed grouped t-test), melittin caused significantly ( $P < .001$ ) greater diacylglyceride and FFA formation

than CTX. Melittin (10  $\mu$ M) was the only toxin causing significant triglyceride breakdown ( $P < .0001$ ). Melittin (2 and 10  $\mu$ M) and CTX (10  $\mu$ M) caused significant ( $P < .01$ ) phospholipid hydrolysis, as determined by the relative reduction of radiolabeled phospholipid. CTX (3  $\mu$ M) caused greater diacylglyceride and FFA formation ( $P < .01$ ) than observed in control cells. CTX at 10  $\mu$ M produced more diacylglyceride ( $P < .01$ ) and FFA ( $P < .001$ ) than at a 3  $\mu$ M concentration. Likewise melittin at the higher (10  $\mu$ M) concentration caused greater diacylglyceride ( $P < .0001$ ) and FFA ( $P < .001$ ) formation than at the lower (2  $\mu$ M) concentration.

The composition of the radioactivity released to the supernatant by 10  $\mu$ M concentrations of melittin and CTX was examined in cultures of human skeletal muscle (Fletcher et al., 1991a). Although the amount of radioactivity was relatively low, it was clear that the distribution was very similar to that retained by the cells. The bulk of the radiolabel was phospholipid (70-80%). Also released at detectable levels were FFA (5-15%), diacylglyceride (6-8%) and triglyceride (4-5%). Altogether the above findings support activation of tissue PLC by CTX and melittin, not activation of PLA<sub>2</sub>.

We next examined whether cultures from patients diagnosed as MH susceptible (MH+) were suitable for use in the toxin studies, since a large number of our studies are conducted with these tissues and since PLC (Foster et al., 1989; Scholz et al., 1991a; b) and triglyceride (Fletcher et al., 1989; 1990b) metabolism have been reported as altered in humans and swine with this disorder. There were no significant differences between the MH- and MH+ groups ( $P > 0.05$ ; two-tailed grouped t-test) in the uptake of radiolabeled linoleic acid into any of the neutral lipids or phospholipids (see Table 17 in Mid-Term Report) in primary cultures of human skeletal muscle (control condition). In general agreement with the results obtained with MH- cultures, there was an increase in diacylglycerol and FFAs in MH+ cultures treated with CTX (see Table 17 in Mid-Term Report), supporting activation of PLC. CTX caused significant, but subtle and likely inconsequential, differences between MH- and MH+ cells in the neutral lipid and phospholipid distribution (see Table 17 in Mid-Term Report), suggesting that the MH+ cells were suitable for use in these toxin studies.

Using [<sup>14</sup>C]ethanolamine to radiolabel the phospholipid head groups in primary cultures of human skeletal muscle resulted in greater than 90% of the incorporated radiolabel being associated with phosphatidylethanolamine and no more than 2% being associated with any other phospholipid (Fletcher and Jiang, 1992). There was also no detectable radiolabel in the neutral lipid fraction. In the absence of toxin there was no significant change in either the phosphoethanolamine or ethanolamine radiolabel in the aqueous phase of the cell extract over the 2 hr incubation (Fletcher and Jiang, 1992). Phosphoethanolamine, a product of PLC activity, was increased to about the same extent in MH- and MH+ cultures following *p*-BPB-treated CTX or *p*-BPB-treated melittin treatment (see Table 18 in Mid-Term Report). Ethanolamine, which could either be a direct product of PLD activity or an indirect product of sequential PLC and phosphoethanolamine phosphatase activities, was elevated two- to three-fold more by melittin than by CTX in these MH- and MH+ primary cell cultures (see Table 18 in Mid-Term Report). The release of phosphoethanolamine was also confirmed in C<sub>2</sub>C<sub>12</sub> cells, but without the concomitant release of ethanolamine (Figure 33).

Again using radiolabeled ethanolamine (see Table 19 in Mid-Term Report), the levels of phosphoethanolamine release induced by CTX and melittin in the absence of pertussis toxin

were comparable to those observed in Table 19 with human skeletal muscle cultures. In contrast, levels of ethanolamine release (see Table 19 in Mid-Term Report) were about one-third to one-fourth the values of the previous study (see Table 18 in Mid-Term Report). Incubation with pertussis toxin (400 ng/ml) for 24 hrs prior to CTX or melittin addition did not significantly alter the uptake of radiolabeled ethanolamine over that period (data not shown). Pertussis toxin did not antagonize the release of either phosphoethanolamine or ethanolamine induced by CTX or melittin (see Table 19 in Mid-Term Report). Indeed, pertussis toxin slightly, but significantly ( $P < 0.05$ ; grouped two-tailed t-test), increased the amount of phosphoethanolamine and ethanolamine released by melittin, but not CTX (see Table 19 in Mid-Term Report). There was no effect of pertussis toxin on the percentage of radiolabel released to the incubation medium by CTX or melittin (see Table 19 in Mid-Term Report). Similar results were obtained in a second identical study (see also Fletcher and Jiang, 1992).

The levels of phosphoethanolamine release induced by CTX and melittin were examined in cells exposed to cholera toxin (1000 ng/ml) in human skeletal muscle cultures (see Table 20 in Mid-Term Report). Incubation with cholera toxin for 24 hrs prior to CTX or melittin addition did not significantly alter the uptake of radiolabeled ethanolamine over that period (data not shown). Cholera toxin did not antagonize the release of either phosphoethanolamine or ethanolamine induced by CTX or melittin (see Table 19 in Mid-Term Report). Like pertussis toxin, cholera toxin slightly, but significantly ( $P < 0.05$ ; grouped two-tailed t-test), increased the amount of phosphoethanolamine released by melittin. However, cholera toxin also increased ( $P < 0.05$ ) the amount of phosphoethanolamine released by CTX. The amounts of ethanolamine released by either CTX or melittin were not significantly affected by cholera toxin. There was no effect of cholera toxin on the percentage of radiolabel released to the incubation medium by CTX, but there was a slight, but significant ( $P < 0.01$ ), decrease in the melittin-induced release of radioactivity into the incubation medium (see Table 19 in Mid-Term Report). Similar results were obtained in a second identical study (see also Fletcher and Jiang, 1992).

The cells used in the cholera toxin study were simultaneously labeled with [ $^{14}\text{C}$ ]linoleic acid to confirm the production of FFAs and diacylglycerol (see Table 19 in Mid-Term Report). Both diacylglycerol and FFA production were significantly increased by CTX and melittin. Diacylglycerol production by CTX ( $P < 0.05$ ) and melittin ( $P < 0.001$ ) was significantly increased by cholera toxin. The production of FFAs was not significantly ( $P > 0.05$ ) affected by cholera toxin for either CTX or melittin (see Table 19 in Mid-Term Report). Therefore, the action of cholera toxin on phosphoethanolamine resembled that on diacylglycerol in these cells.

Our more recent studies have demonstrated that under conditions in which PLC activity was increased by melittin and CTX (monitoring release of FFA and diacylglycerol), there was no release of phosphocholine despite extensive labeling of phosphatidylcholine, suggesting that the activated PLC had a substrate specificity for phosphatidylethanolamine and does not readily hydrolyze phosphatidylcholine (data not shown). The preferred hydrolysis of PE over PC by melittin was also demonstrated by following the decrease in radiolabel in the Toxin X studies referred to below (see associated figure).

Since the effects of melittin and CTX on  $\text{Ca}^{2+}$  release could be mediated through the

production of FFAs, which have effects similar to those of the toxins (Fletcher et al., 1990b), we examined the time course of melittin action. The time course of CTX action has been published (Fletcher et al., 1991b). Two separate experiments with melittin (10  $\mu$ M) were conducted involving release of radioactivity into the bathing medium (see Figure 15 in Mid-Term Report and Fletcher and Jiang, 1993). Different levels of total radioactivity released are common in our system, as are different amounts of FFA formation, diacylglycerol production and phosphoethanolamine release. In both studies in primary cultures of human skeletal muscle it was obvious that significant levels of radioactivity are released at early (1 min) time points, consistent with a rapid effect of these toxins on this yet-to-be-defined exocytotic (apparently) process (see Figure 15 in Mid-Term Report). The release of diacylglycerol and FFA for each of these experiments was also monitored (see Figures 16 and 17 in Mid-Term Report and Fletcher and Jiang, 1993). In both studies significant levels of at least one of these indicators of PLC activity was elevated within one min, consistent with the rapid action of melittin on  $\text{Ca}^{2+}$  release. It should be remembered that the effects of melittin on  $\text{Ca}^{2+}$  release were monitored at a 0.1  $\mu$ M concentration of melittin. Such low levels of toxin have not been examined with regard to PLC activity activation in cell culture. Higher concentrations of melittin (2  $\mu$ M) do cause significant activation of PLC, but these effects are difficult to quantitate at such low concentrations of toxin. Further evidence for a rapid effect of melittin on PLC activity is provided in the second study in which phosphoethanolamine release was also significantly elevated within one min of toxin exposure (see Figure 18 in Mid-Term Report and Fletcher and Jiang, 1993). Ethanol release [an indicator of phospholipase D (PLD) activity] was not elevated at early time points and was never a major component of toxin action, suggesting that this was not activation of a PLD, but rather resulted from phosphatase activity on phosphoethanolamine. Also, propranolol (200 or 400  $\mu$ M), an antagonist of phosphatidate phosphohydrolase, has no effect on the action of melittin (10  $\mu$ M), suggesting that the production of diacylglycerol by the toxin is not through activation of PLD (data not shown). In all, these studies are at least preliminary evidence that activation of PLC by toxins may indirectly lower the TCICR by an action of the products of lipolysis.

Since myotoxin *a* caused  $\text{Ca}^{2+}$  release (Yudkowsky et al., 1994), we tested whether this toxin might also be acting through activation of PLC activity. Four separate studies were conducted in: 1) nondifferentiated NB41A3 cells; 2) differentiated NB41A3 cells; 3) differentiated primary cultures of human skeletal muscle and; 4)  $\text{C}_2\text{C}_{12}$  cells. In all four cases there was no detectable effect of myotoxin *a* on release of radiolabel into the incubation medium, or the production of any products of  $\text{PLA}_2$ , PLC, PLD, or triglyceride breakdown. Therefore, although myotoxin *a* exerts similar effects on the  $\text{Ca}^{2+}$  release channel, it differs from CTX and melittin in regard to activation of PLC.

Since many aspects of lipid metabolism have been reported to be  $\text{Ca}^{2+}$  dependent, it is possible that a direct stimulation of  $\text{Ca}^{2+}$  release by the toxins could account for their effects on lipid metabolism. However, it is unlikely that melittin and CTX would specifically activate PLC and that myotoxin *a* would specifically activate  $\text{PLA}_2$  activity indirectly through a common action on  $\text{Ca}^{2+}$  release. In addition, in two separate studies we treated human cell cultures with EDTA (2 or 10 mM) and a  $\text{Ca}^{2+}$ -free medium to eliminate  $\text{Ca}^{2+}$  influx and added ruthenium red to block  $\text{Ca}^{2+}$  release by the toxins. Under these conditions, melittin (2 or 10



$\mu\text{M}$ ) did not induce release of radioactivity into the medium above control levels (see Figure 22 in Mid-Term Report and Fletcher and Jiang, 1993). However, note that the  $\text{Ca}^{2+}$ -free conditions promoted release of radiolabel in control preparations. Since diacylglycerol production and FFA release (see Figure 23 in Mid-Term Report and Fletcher and Jiang, 1993) were unaffected by  $\text{Ca}^{2+}$ -free medium, this means that release of radiolabel into the medium is independent of  $\text{PLA}_2$  and PLC activation and suggests an additional action of melittin. In contrast to the lack of effects of melittin on release of radiolabel into the incubation medium under  $\text{Ca}^{2+}$ -free conditions, the toxin increases both diacylglycerol and FFA (see Figure 23 in Mid-Term Report and Fletcher and Jiang, 1993). Therefore, melittin activates PLC activity through a  $\text{Ca}^{2+}$ -independent mechanism. Attempts to repeat these studies in the mouse cell line failed because the EDTA-containing medium lifted the cells off the wells (not completely unexpected).

***Effects of Convulxin, Bothropstoxin, Thionin and an Unidentified Toxin from Crotalus viridis viridis Venom on Lipid Metabolism in Cell Culture***

CTXs and melittin activate tissue PLC activity and convulxin has been reported to activate an  $\text{PIP}_2$ -specific PLC in platelets. Due to these apparent similarities in the toxins, we compared convulxin to melittin in activation of PLC in primary cultures of human skeletal muscle and in differentiated and nondifferentiated cell lines (Tables 7-8). If convulxin activates PLC activity in these cell lines, then it acts by a mechanism very different from melittin and has a specificity for a minor substrate (e.g.  $\text{PIP}_2$ ).

The lipid hydrolyzing activities of a myotoxic  $\text{PLA}_2$  (BthTx from *Bothrops jararacussa* venom) and plant thionin were also examined. BthTx possesses  $\text{PLA}_2$  activity in cell culture systems (Tables 9-11, note in particular FFAs and LPC) that previously was undetected by other investigators using purified substrates. BthTx  $\text{PLA}_2$  activity is lower than that of the *Naja naja atra*  $\text{PLA}_2$  (data in duplicate not shown for 100 and 1000 nM BthTX) and is qualitatively different, resulting in a large build-up of PA/CL (Tables 9-11). While BthTx might slightly increase DG production through activation of PLC activity (Tables 9 and 11), this action is far less than that of melittin (Table 11). Thionin is still a puzzle. Thionin does produce DG (product of PLC activity not observed with  $\text{PLA}_2$ ), but not to the same extent as melittin (Tables 12 and 13). Thionin also produces far less FFA than melittin (Tables 12 and 13). We did not observe the elevated LPC reported by others that would suggest activation of a cellular  $\text{PLA}_2$  (data not shown).

We found that one lot of toxin that we had originally mistakenly identified as myotoxin a, based on information provided by our commercial source, was instead a crude fraction from *Crotalus viridis viridis* that was an early step in the purification of myotoxin a. This necessitated repeating a number of studies to establish what activities were truly due to myotoxin a and what were due to another component in the crude fraction. We used purified myotoxin a obtained from Dr. Charlotte Ownby and found that this toxin did not activate lipase activity in cell cultures when tested in the same cultures and at the same time as the fraction containing what we now refer to as "Toxin X".

Toxin X is a unique toxin as it appears to specifically activate tissue  $\text{PLA}_2$  activity. Toxin X does not cause release of radioactivity into the incubation medium (Figure 34) in cells radiolabeled with choline in addition to linoleic acid. Also, in the same cells free fatty acids, but not diacylglycerol are released (Figure 34). These findings support a difference between

Toxin X and cardiotoxin or melittin and suggest  $PLA_2$  activation by Toxin X.

We compared Toxin X to melittin in the mouse  $C_2C_{12}$  cell line preradiolabeled with [ $^{14}C$ ]linoleic acid and either [ $^{14}C$ ]choline or [ $^{14}C$ ]ethanolamine. As in Figure 34, there was release of radiolabel into the incubation medium with melittin, but not Toxin X (Figure 35). Since lysophosphatidylethanolamine (LPE) migrates on our 1-D TLC plates with approximately the same  $R_f$  as phosphatidylcholine (PC) and phosphatidylinositol (PI), we used the sum of PC+PI to indicate production of LPE (PC+PI+LPE - PC+PI). Melittin exhibits no significant change in PC+PI, as it is activating PLC with a preference for PE (LPE is not produced by PLC). In contrast, Toxin X produces LPE to about the same extent as PE decreases whether the cells are preradiolabeled with choline or ethanolamine (Figure 35). The production of FFAs and DG by these toxins in these cells (Figure 35) was similar to Figure 34. These results support activation of cellular  $PLA_2$  by toxin X, as venom  $PLA_2$  would have produced LPC, not LPE. No LPC was detectable (data not shown).

The release of radioactivity into the incubation medium is commonly used as an indicator of activation of cellular  $PLA_2$ s. However, we have found that the same amount of radioactivity is released to the incubation medium whether or not PLC is activated (Figure 36) and no radioactivity is released to the incubation medium if  $PLA_2$  is activated, as demonstrated for Toxin X (Figure 35). Therefore, release of radioactivity into the medium may be nothing more than nonlipase-mediated, nonspecific detergent interactions of the toxins with the membrane, releasing membrane into the medium.

In conclusion, even highly purified venom fractions of CTXs and melittin contain trace amounts of venom  $PLA_2$ . The  $PLA_2$  activity contaminating these fractions can be eliminated by either treating the fraction with *p*-BPB, or, in the case of melittin, by using synthetic toxin. Snake venom CTXs and bee venom melittin activate tissue PLC activity (not tissue  $PLA_2$ ) within 1 min and the products of this activity continue to be generated for hours. The PLC activity is directed preferentially toward PE over PC. At high concentrations (10  $\mu$ M) melittin also activates triglyceride lipolysis. Both CTX and melittin release cell membrane fragments into the incubation medium. The release of membrane fragments into the incubation medium and activation of PLC by these toxins is mostly independent of extracellular  $Ca^{2+}$  and is not antagonized by pertussis or cholera toxin. A fraction derived from *Crotalus viridis viridis*, initially mistaken for myotoxin  $\alpha$ , appears to activate tissue  $PLA_2$  activity. Convulxin, which appears to activate a PI-hydrolyzing PLC, did not significantly affect lipid metabolism in our system, suggesting it may be specific for  $PIP_2$ . Thionin appears to be similar to CTX and melittin in action. Bothropstoxin has a unique  $PLA_2$  activity.

## TABLES

TABLE 1. Effects of Mojave toxin and its subunits on Ch uptake in mouse brain synaptosomes. Toxins (100 nM) were incubated with or without BSA (0.5%) for 60 min at 25°C and choline uptake was subsequently determined at 37°C.

	Choline Uptake	
	- BSA	+ BSA
	(% control; mean $\pm$ SD; n = 3)	
Whole toxin	36 $\pm$ 5	69 $\pm$ 9
Basic subunit	49 $\pm$ 2	73 $\pm$ 10
Acidic subunit	99 $\pm$ 10	96 $\pm$ 10

TABLE 2. Effects of a 100 nM concentration of *Naja naja atra* PLA<sub>2</sub>,  $\beta$ -Butx and scutoxin on Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent choline uptake. Toxins were incubated with or without BSA (0.5%) for 60 min at 25°C and choline uptake was subsequently determined in the absence or presence of Na<sup>+</sup> at 37°C. Note: Na<sup>+</sup>- independent choline uptake was about 35% of the uptake in the presence of Na<sup>+</sup>.

<u>Toxin</u>	No Na <sup>+</sup>		Na <sup>+</sup> Present	
	No BSA	BSA 0.5%	No BSA	BSA 0.5%
<i>N.n.a.</i> PLA <sub>2</sub>	34 $\pm$ 10	94 $\pm$ 8*	41 $\pm$ 4	105 $\pm$ 0*
$\beta$ -Butx	58 $\pm$ 12	65 $\pm$ 4	57 $\pm$ 1	54 $\pm$ 4@
Scutoxin	20 $\pm$ 5	44 $\pm$ 7*	14 $\pm$ 1	26 $\pm$ 3*@

\*Difference between No BSA and BSA (P < 0.002 ).

@Difference between No Na<sup>+</sup> and Na<sup>+</sup> present (P < 0.03).

TABLE 3. Reversibility of  $\beta$ -Butx and scutoxin binding, as regards stimulation of ACh release. Comparison of incubation of synaptosomes for 60 min with  $\beta$ -Butx or scutoxin (100 nM) to incubation for 1 min with toxin and 59 additional min with no toxin present.

	1 min with toxin,	
	59 min without toxin	60 min with toxin
	ACh Release [% Control; mean $\pm$ SD (3)]	
$\beta$ -Butx	126 $\pm$ 4	124 $\pm$ 3
Scutoxin	139 $\pm$ 3	140 $\pm$ 4

TABLE 4. Stimulation of ACh release, as determined in mouse brain synaptosomes by HPLC and electrochemical detection. Conditions: 25°C, 60 min.

Toxin	ACh Release	
	-BSA	+BSA
	(% control)	
<i>Naja naja atra</i> PLA <sub>2</sub>	130	86
β-Butx	150	128
Pseudexin	279	130

Values are the average of duplicate determinations.

TABLE 5. Hydrolysis of radiolabeled nerve cell cultures by PSNTXs and nonPSNTX PLA<sub>2</sub>s. Percentage of hydrolysis by nonneurotoxic and neurotoxic PLA<sub>2</sub>s of three phospholipids (PC, PS, PE) based on the mean values for each phospholipid relative to SM (not hydrolyzed by PLA<sub>2</sub>s) for control and toxin-treated preparations (3 NB41A3; 3 PC12). The hydrolysis of the three PLs were compared statistically within each toxin treatment.

Toxin	n	PC	PS	PE
		% Hydrolysis (mean ± SD)		
<i>Nna</i> PLA <sub>2</sub>	5	72 ± 10	54 ± 30	91 ± 9 <sup>b</sup>
<i>Nnk</i> PLA <sub>2</sub>	3	74 ± 14 <sup>a</sup>	51 ± 15	89 ± 4 <sup>b</sup>
β-Butx	5	68 ± 18 <sup>a</sup>	37 ± 26	62 ± 32
Pseudexin B	4	82 ± 16	82 ± 11	86 ± 19
Scutoxin	3	86 ± 5	84 ± 9	97 ± 4

<sup>a</sup>Different from PS (P<0.05) by one-way ANOVA (repeated measures) and Sheffe test.

<sup>b</sup>Different from PS (P<0.01) by one-way ANOVA (repeated measures) and Sheffe test.

TABLE 6. Effects of myotoxin a (10 μM) on the Ca<sup>2+</sup> pump in equine heavy sarcoplasmic reticulum fractions. Since Ca<sup>2+</sup> uptake, as determined in this assay, is the net result of Ca<sup>2+</sup> release and Ca<sup>2+</sup> uptake, ruthenium red was added to block the Ca<sup>2+</sup> release channel to just monitor Ca<sup>2+</sup> uptake.

	No ruthenium red	Ruthenium red (10 μM)
	Ca <sup>2+</sup> Uptake (nmol Ca <sup>2+</sup> /mg.sec)	
Control	18.1 ± 4.4	24.1 ± 0.9
Myotoxin a	10.5 ± 1.5	29.0 ± 5.4
	P<0.05	P=0.20 (n.s.)

All values are the mean ± SD (3). Control vs. myotoxin a analyzed by t-test.

TABLE 7A. Effects of convulxin on lipid metabolism in muscle - release of radioactivity into the incubation buffer. Primary cultures of human skeletal muscle were preradiolabeled with [ $^{14}\text{C}$ ]linoleic acid (18:2; 10  $\mu\text{M}$ ) and [ $^{14}\text{C}$ ]ethanolamine (10  $\mu\text{M}$ ) for three days.

Percent of total radiolabel released to the medium

	mean $\pm$ SD (n = 3)
Control	4.9 $\pm$ 0.4
Convulxin 50 nM	4.8 $\pm$ 0.5
Melittin 10 $\mu\text{M}$	24 $\pm$ 3*

\*Different from control ( $P < 0.0001$ ) when all three conditions are compared by a one-way ANOVA and Shefe test.

TABLE 7B. Effects of convulxin on lipid metabolism in muscle - neutral lipid TLC separation. Primary cultures of human skeletal muscle were preradiolabeled with [ $^{14}\text{C}$ ]linoleic acid (18:2; 10  $\mu\text{M}$ ) and [ $^{14}\text{C}$ ]ethanolamine (10  $\mu\text{M}$ ) for three days.

	PL	DG	FFA	TG	CHE
	(% distribution of label; mean $\pm$ SD, n = 3)				
Control	91 $\pm$ 0	0.49 $\pm$ 0.16	0.14 $\pm$ 0.25	7.7 $\pm$ 0.3	0.47 $\pm$ 0.03
Convulxin 50 nM	89 $\pm$ 3	0.26 $\pm$ 0.24	0.00 $\pm$ 0.00	9.7 $\pm$ 2.9	0.88 $\pm$ 0.36
Melittin 10 $\mu\text{M}$	85 $\pm$ 3	2.88 $\pm$ 0.09*	2.70 $\pm$ 0.39*	8.9 $\pm$ 2.2	0.57 $\pm$ 0.05

Abbreviations: PL, phospholipid; DG, diacylglycerol; FFA, free fatty acids; TG, triacylglycerol; CHE, cholesterol esters.

\*Different from control ( $P < 0.0001$ ) when all three conditions are compared by a one-way ANOVA and Shefe test.

TABLE 7C. Effects of convulxin on lipid metabolism in muscle - phospholipid TLC separation. Note that PA and CL were integrated as a single peak since they were not completely resolved from one another. Primary cultures of human skeletal muscle were preradiolabeled with [ $^{14}\text{C}$ ]linoleic acid (18:2; 10  $\mu\text{M}$ ) and [ $^{14}\text{C}$ ]ethanolamine (10  $\mu\text{M}$ ) for three days.

	SM	PC	PI	PS	PE	PA/CL
	(% distribution of label; mean $\pm$ SD, n = 3)					
Control	1.2 $\pm$ 0.1	25 $\pm$ 1	3.6 $\pm$ 0.9	4.3 $\pm$ 0.1	60 $\pm$ 1	5.7 $\pm$ 0.3
Convulxin 50 nM	1.1 $\pm$ 0.1	28 $\pm$ 2	3.4 $\pm$ 0.3	3.2 $\pm$ 0.3	58 $\pm$ 1	6.3 $\pm$ 1.6
Melittin 10 $\mu\text{M}$	1.6 $\pm$ 0.3	30 $\pm$ 4	4.2 $\pm$ 1.1	3.5 $\pm$ 0.8	55 $\pm$ 2*	5.4 $\pm$ 0.4

Abbreviations: SM sphingomyelin; PC phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; CL, cardiolipin.

\*Different from control ( $P < 0.05$ ) when all three conditions are compared by a one-way ANOVA and Shefe test.

TABLE 8A. Effects of convulxin on lipid metabolism in nerve - release of radioactivity into the incubation buffer. PC12 cells were preradiolabeled with [ $^{14}$ C]linoleic acid (18:2; 10  $\mu$ M) and [ $^{14}$ C]ethanolamine (10  $\mu$ M) for one day. Cells were incubated with toxin (2 hrs; 37°C) in F-10 plus  $\text{Ca}^{2+}$  (2 mM).

Percent of total radiolabel released to the medium  
mean  $\pm$  SD (n = 3)

*Undifferentiated Cells*

Control 6.2  $\pm$  0.8

Convulxin 25 nM 5.8  $\pm$  0.3

*Differentiated Cells* - NGF (7S; 100 ng/ml; 6 days)

Control 6.0  $\pm$  0.4

Convulxin 25 nM 6.2  $\pm$  0.7

Convulxin 50 nM 4.9  $\pm$  0.3

Melittin 5  $\mu$ M 29  $\pm$  0.4\*

\*Different from control ( $P < 0.0001$ ) when all conditions are compared by a one-way ANOVA and Shefe test.

TABLE 8B. Effects of convulxin on lipid metabolism in nerve - neutral lipid TLC separation. PC12 cells were preradiolabeled with [ $^{14}$ C]linoleic acid (18:2; 10  $\mu$ M) and [ $^{14}$ C]ethanolamine (10  $\mu$ M) for one day.

PL DG FFA TG  
(% distribution of label; mean  $\pm$  SD, n = 3)

*Undifferentiated Cells*

Control 97  $\pm$  0 1.5  $\pm$  0.0 0.61  $\pm$  0.11 1.2  $\pm$  0.2

Convulxin 25 nM 97  $\pm$  0 1.2  $\pm$  0.1\* 0.54  $\pm$  0.07 0.9  $\pm$  0.1

*Differentiated Cells* - NGF (7S; 100 ng/ml; 6 days)

Control 96  $\pm$  0 1.4  $\pm$  0.0 0.76  $\pm$  0.08 1.5  $\pm$  0.2

Convulxin 25 nM 97  $\pm$  0 1.3  $\pm$  0.1 0.65  $\pm$  0.04 1.1  $\pm$  0.3

Convulxin 50 nM 96  $\pm$  0 1.5  $\pm$  0.1 0.58  $\pm$  0.07 1.6  $\pm$  0.3

Melittin 5  $\mu$ M 94  $\pm$  1\*\* 2.2  $\pm$  0.1\*\* 2.67  $\pm$  0.24\*\* 1.6  $\pm$  0.4

Abbreviations: see above.

Different from control \*( $P < 0.01$ ) or \*\*( $P < 0.0001$ ) one-way ANOVA and Shefe test.

TABLE 8C. Effects of convulxin on lipid metabolism in nerve - phospholipid TLC separation. PC12 cells were preradiolabeled with [ $^{14}\text{C}$ ]linoleic acid (18:2; 10  $\mu\text{M}$ ) and [ $^{14}\text{C}$ ]ethanolamine (10  $\mu\text{M}$ ) for one day.

	SM	PC	PI	PS	PE	PA	CL
(% distribution of label; mean $\pm$ SD, n = 3)							
<i>Undifferentiated Cells</i>							
Control	0.2 $\pm$ 0.4	14 $\pm$ 1	4.2 $\pm$ 0.2	3.4 $\pm$ 0.2	73 $\pm$ 1	3.5 $\pm$ 1.2	1.4 $\pm$ 1.6
Convulxin 25 nM	0.6 $\pm$ 0.1	14 $\pm$ 0	4.5 $\pm$ 0.2	3.5 $\pm$ 0.3	73 $\pm$ 1	2.0 $\pm$ 1.1	2.1 $\pm$ 1.9
<i>Differentiated Cells - NGF (7S; 100 ng/ml; 6 days)</i>							
Control	0.4 $\pm$ 0.7	18 $\pm$ 1*	4.2 $\pm$ 0.5	4.1 $\pm$ 0.9	69 $\pm$ 1*	2.1 $\pm$ 0.3	2.6 $\pm$ 0.3
Convulxin 50 nM	0.6 $\pm$ 0.0	16 $\pm$ 0	3.9 $\pm$ 0.2	3.5 $\pm$ 0.4	71 $\pm$ 1	1.6 $\pm$ 0.1	2.5 $\pm$ 0.1
Melittin 5 $\mu$ M	0.3 $\pm$ 0.3	17 $\pm$ 1	3.8 $\pm$ 0.1	3.6 $\pm$ 0.3	71 $\pm$ 0	2.5 $\pm$ 1.2	1.8 $\pm$ 1.6

Abbreviations: see above.

\*Differentiated control different ( $P < 0.01$ ) from undifferentiated control.

TABLE 9. Effects of *Naja naja atra* PLA<sub>2</sub> (100 nM) and BthTX (10  $\mu\text{M}$ ) on lipid metabolism. Nondifferentiated and differentiated neuroblastoma (NB41A3) cells were first incubated with or without sodium butyrate (0.5 mM) for 6 days, then were radiolabeled with [ $^{14}\text{C}$ ]linoleic acid (18:2; 10  $\mu\text{M}$ ) for 3 days (sodium butyrate still present). After washing off excess radiolabel the cells were incubated in F-10 growth medium (no serum; no BSA) supplemented with Ca<sup>2+</sup> (2 mM) with or without toxin for 2 hr at 37°C. All values are mean  $\pm$  SD (n = 3).

	Nondifferentiated			Differentiated		
	Control	PLA <sub>2</sub>	BthTX	Control	PLA <sub>2</sub>	BthTX
	(% of total cell radioactivity)					
Release	2.3 $\pm$ 0.5	2.1 $\pm$ 0.2	27 $\pm$ 2 <sup>c,f</sup>	2.1 $\pm$ 0.2	1.9 $\pm$ 0.1	14 $\pm$ 4 <sup>b,c</sup>
Lipid	(% of total lipid)					
PL	67 $\pm$ 6	55 $\pm$ 6	22 $\pm$ 1 <sup>c,f</sup>	43 $\pm$ 4	42 $\pm$ 3	28 $\pm$ 6 <sup>a,d</sup>
DG	1.3 $\pm$ 0.1	1.2 $\pm$ 0.2	1.8 $\pm$ 0.2 <sup>d</sup>	1.2 $\pm$ 0.1	1.0 $\pm$ 0.1	1.4 $\pm$ 0.2 <sup>d</sup>
FFA	0.9 $\pm$ 0.3	6.7 $\pm$ 0.9 <sup>c</sup>	35 $\pm$ 1 <sup>c,e</sup>	0.3 $\pm$ 0.2	3.8 $\pm$ 0.5	14 $\pm$ 6 <sup>a,d</sup>
TG	30 $\pm$ 7	37 $\pm$ 6	40 $\pm$ 1	55 $\pm$ 4	54 $\pm$ 3	57 $\pm$ 3
Phospholipid	(% of total phospholipid)					
LPC	0.3 $\pm$ 0.1	1.4 $\pm$ 0.2	17 $\pm$ 3 <sup>c,e</sup>	0.4 $\pm$ 0.1	2.7 $\pm$ 0.2	14 $\pm$ 6 <sup>a,d</sup>
SM	0.3 $\pm$ 0.2	0.4 $\pm$ 0.2	1.0 $\pm$ 0.4	0.7 $\pm$ 0.1	0.9 $\pm$ 0.1	1.2 $\pm$ 0.3
PC	52 $\pm$ 3	48 $\pm$ 2	17 $\pm$ 2 <sup>c,e</sup>	54 $\pm$ 1	52 $\pm$ 2	35 $\pm$ 8 <sup>b,d</sup>
PI(+LPE)	9.4 $\pm$ 1.0	8.6 $\pm$ 0.2	9.1 $\pm$ 2.6	9.2 $\pm$ 1.5	11 $\pm$ 1	10 $\pm$ 0
PS	1.6 $\pm$ 0.7	2.2 $\pm$ 0.5	1.9 $\pm$ 1.0	2.3 $\pm$ 0.3	2.9 $\pm$ 0.7	2.4 $\pm$ 0.1
PE	24 $\pm$ 1	20 $\pm$ 1 <sup>a</sup>	17 $\pm$ 1 <sup>b,d</sup>	21 $\pm$ 1	18 $\pm$ 1 <sup>a</sup>	18 $\pm$ 1 <sup>a</sup>
PA(+CL)	13 $\pm$ 1	19 $\pm$ 3	38 $\pm$ 9 <sup>b,d</sup>	13 $\pm$ 1	13 $\pm$ 1	20 $\pm$ 3 <sup>b,d</sup>
	(cont.)					

## (Table 9 - Cont.)

Different from control by ANOVA and Scheffe test \* $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ .

Different from *Naja naja atra* PLA<sub>2</sub> by ANOVA and Scheffe test <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ .

PLA<sub>2</sub> = *Naja naja atra* PLA<sub>2</sub> (100 nM)

BthTX = bothropstoxin from *Bothrops jararacussa* venom (10  $\mu$ M)

Abbreviations: CHE, cholesterol esters; CL, cardiolipin; CTX, cardiotoxin; DG, diacylglyceride; FFA, free fatty acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PS, phosphatidylserine; LPC, 1.7%, SM, sphingomyelin; TG, triacylglyceride.

Note: melittin control (n = 1): *Nondifferentiated cells*. Release 59%; PL 45%; DG 6.4%; FFA 20%; TG 29%. All phospholipids similar to control, except PI 11%, PE 18%, PA 18%. *Differentiated cells*. Release 40%, PL 46%, DG 7.1%, FFA 18%, TG 29%. All phospholipids similar to control, except LPC 2.9%, PS 3.7%, PE 14%, PA 14%.

TABLE 10. Effects of melittin (10  $\mu$ M) and BthTX (10  $\mu$ M) on lipid metabolism.

Differentiated pheochromocytoma (PC12) cells were first incubated with or without NGF (100  $\mu$ g/ml) for 6 days, then were radiolabeled with [<sup>14</sup>C]linoleic acid (18:2; 10  $\mu$ M) for 3 days (NGF still present). After washing off excess radiolabel the cells were incubated in F-10 growth medium (no serum; no BSA) supplemented with Ca<sup>2+</sup> (2 mM) with or without toxin for 2 hr at 37°C. All values are mean  $\pm$  SD (n = 3).

	Differentiated			Nondifferentiated	
	Control	Melittin	BthTX	Control	BthTX
	(% of total cell radioactivity)				
Release	0.7 ± 0.1	9.2 ± 1.2 <sup>c,e</sup>	1.9 ± 0.4	1.7 ± 0.2	2.5 ± 0.2
Lipid	(% of total lipid)				
PL	88 ± 4	80 ± 1 <sup>a</sup>	83 ± 2	90 ± 2	66 ± 1
DG	3.5 ± 0.8	5.1 ± 0.6 <sup>c</sup>	2.7 ± 0.6	1.7 ± 0.3	1.8 ± 0.2
FFA	1.4 ± 0.5	9.3 ± 1.1 <sup>c,f</sup>	3.6 ± 0.4 <sup>a</sup>	2.8 ± 1.4	27 ± 1
TG	7.4 ± 2.4	5.4 ± 0.4 <sup>d</sup>	10 ± 1	5.1 ± 0.9	5.2 ± 0.7
Phospholipid	(% of total phospholipid)				
LPC				0.3 ± 0.4	4.7 ± 0.8
SM				1.1 ± 0.1	1.4 ± 0.2
PC				36 ± 1	30 ± 1
PI(+LPE)				8.9 ± 0.7	7.6 ± 0.5
PS				8.3 ± 0.8	5.5 ± 0.2
PE				27 ± 0	26 ± 1
PA(+CL)				18 ± 1	25 ± 3

Different from control by ANOVA and Scheffe test \* $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ .

Different from bothropstoxin by ANOVA and Scheffe test <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.001$ .

BthTX = bothropstoxin from *Bothrops jararacussa* venom (10  $\mu$ M)

Abbreviations: See Table 1.



TABLE 11. Effects of *Naja naja atra* PLA<sub>2</sub> (100 nM and 10 μM), melittin (10 μM) and BthTx (10 μM) on lipid metabolism. Nondifferentiated neuroblastoma (NB41A3) cells were first incubated for 5 days, then were radiolabeled with [<sup>14</sup>C]linoleic acid (18:2; 10 μM) for 3 days. After washing off excess radiolabel the cells were incubated in F-10 growth medium (no serum; no BSA) supplemented with Ca<sup>2+</sup> (2 mM) with or without toxin for 2 hr at 37°C. All values are mean ± SD (n = 3). For BthTx the mean of two determinations is shown.

	Control	PLA <sub>2</sub> (100 nM)	PLA <sub>2</sub> (10 μM) (% of total radioactivity)	Melittin (10 μM)	BthTx
Release	1.6 ± 0.2	1.7 ± 0.3	2.8 ± 0.2	15 ± 3 <sup>c,i</sup>	14
Lipid		(% of total lipid)			
PL	46 ± 5	33 ± 1 <sup>a</sup>	17 ± 5 <sup>c,e</sup>	38 ± 3 <sup>i</sup>	16
DG	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	5.4 ± 0.6 <sup>c,f,i</sup>	2.3
FFA	1.2 ± 0.8	8.3 ± 0.3 <sup>a</sup>	32 ± 3 <sup>c,f</sup>	27 ± 3 <sup>c,f</sup>	31
TG	51 ± 6	58 ± 1	50 ± 2	30 ± 2 <sup>c,f,i</sup>	50
Phospholipid			(% of total phospholipid)		
LPC	0.4 ± 0.4	2.5 ± 0.5	25 ± 11 <sup>b,d</sup>	n.d.	22
SM	1.1 ± 0.4	0.8 ± 0.2	1.5 ± 0.3	n.d.	1.8
PC	57 ± 1	56 ± 1	36 ± 16	n.d.	19
PI(+LPE)	5.7 ± 0.8	6.9 ± 0.1	9.4 ± 1.2 <sup>b,d</sup>	n.d.	8.6
PS	1.4 ± 0.8	2.1 ± 0.4	3.1 ± 0.4 <sup>a</sup>	n.d.	3.9
PE	22 ± 0	18 ± 1 <sup>a</sup>	6.9 ± 1.4 <sup>c,f</sup>	n.d.	9.1
PA(+CL)	13 ± 0	14 ± 0	18 ± 5	n.d.	36

Different from control by ANOVA and Scheffe test <sup>a</sup>P<0.05; <sup>b</sup>P<0.01; <sup>c</sup>P<0.001.

Different from *Naja naja atra* PLA<sub>2</sub> (100 nM) by ANOVA and Scheffe test <sup>d</sup>P<0.05; <sup>e</sup>P<0.01; <sup>f</sup>P<0.001.

Different from *Naja naja atra* PLA<sub>2</sub> (10 μM) by ANOVA and Scheffe test <sup>a</sup>P<0.05; <sup>b</sup>P<0.01; <sup>c</sup>P<0.001.

PLA<sub>2</sub> = *Naja naja atra* PLA<sub>2</sub> (100 nM)

Abbreviations: See above; n.d. = not determined.

TABLE 12. Effects of melittin (10  $\mu$ M) and thionin (10  $\mu$ M) on lipid metabolism in differentiated NB41A3 cells in culture. Cells were incubated one day, then incubated with sodium butyrate (0.5 mM) for 5 days, then were radiolabeled with [ $^{14}$ C]linoleic acid (18:2; 10  $\mu$ M) for 2 days (butyrate still present). After washing off excess radiolabel the cells were incubated in F-10 growth medium (no serum; no BSA) supplemented with  $\text{Ca}^{2+}$  (2 mM) with or without toxin for 2 hr at 37°C. All values are mean  $\pm$  SD (n = 3).

	Control	Thionin	Melittin
	(% of total cell radioactivity)		
Release	2.0 $\pm$ 0.3	15 $\pm$ 1 <sup>c</sup>	36 $\pm$ 2 <sup>c,f</sup>
Lipid	(% of total lipid)		
PL	45 $\pm$ 2	45 $\pm$ 1	43 $\pm$ 0
DG	1.2 $\pm$ 0.2	3.1 $\pm$ 0.2 <sup>c</sup>	7.5 $\pm$ 0.3 <sup>c,f</sup>
FFA	0.3 $\pm$ 0.5	3.6 $\pm$ 0.8 <sup>b</sup>	24 $\pm$ 1 <sup>c,f</sup>
TG	53 $\pm$ 2	49 $\pm$ 0 <sup>b</sup>	26 $\pm$ 0 <sup>c,f</sup>

Different from control by ANOVA and Scheffe test \*P < 0.05; <sup>b</sup>P < 0.01; <sup>c</sup>P < 0.001.

Different from thionin by ANOVA and Scheffe test <sup>d</sup>P < 0.05; <sup>e</sup>P < 0.01; <sup>f</sup>P < 0.001.

TABLE 13. Effects of melittin (10  $\mu$ M) and thionin (10  $\mu$ M) on lipid metabolism in nondifferentiated NB41A3 cells in culture. Cells were first incubated for 5 days, then were radiolabeled with [ $^{14}$ C]linoleic acid (18:2; 10  $\mu$ M) for 2 days. After washing off excess radiolabel the cells were incubated in F-10 growth medium (no serum; no BSA) supplemented with  $\text{Ca}^{2+}$  (2 mM) with or without toxin for 2 hr at 37°C. All values are mean  $\pm$  SD (n = 4).

	Control	Thionin	Melittin
	(% of total cell radioactivity)		
Release	1.8 $\pm$ 0.0	6.0 $\pm$ 0.6 <sup>c</sup>	11 $\pm$ 0 <sup>c,f</sup>
Lipid	(% of total lipid)		
PL	49 $\pm$ 6	49 $\pm$ 3	53 $\pm$ 2
DG	1.3 $\pm$ 0.2	2.5 $\pm$ 0.2 <sup>c</sup>	6.4 $\pm$ 0.4 <sup>c,f</sup>
FFA	0.8 $\pm$ 0.2	1.4 $\pm$ 0.1 <sup>**</sup>	25 $\pm$ 1 <sup>c,f</sup>
TG	48 $\pm$ 6	47 $\pm$ 3	16 $\pm$ 1 <sup>c,f</sup>

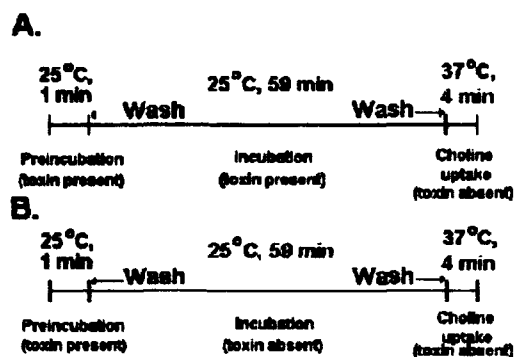
Different from control by ANOVA and Scheffe test \*P < 0.05; <sup>b</sup>P < 0.01; <sup>c</sup>P < 0.001.

Different from thionin by ANOVA and Scheffe test <sup>d</sup>P < 0.05; <sup>e</sup>P < 0.01; <sup>f</sup>P < 0.001.

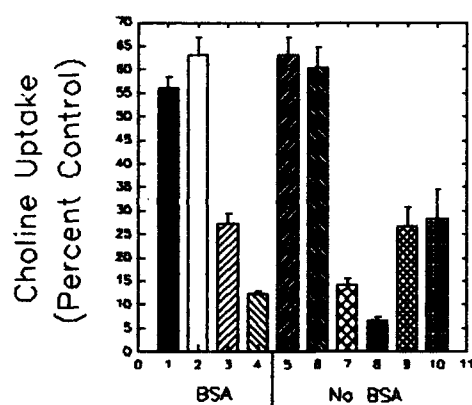
**\*\*High relative variance of melittin masked FFA effect of thionin by ANOVA/Sheffe.**

A t-test between control and thionin is significant (P < 0.01)

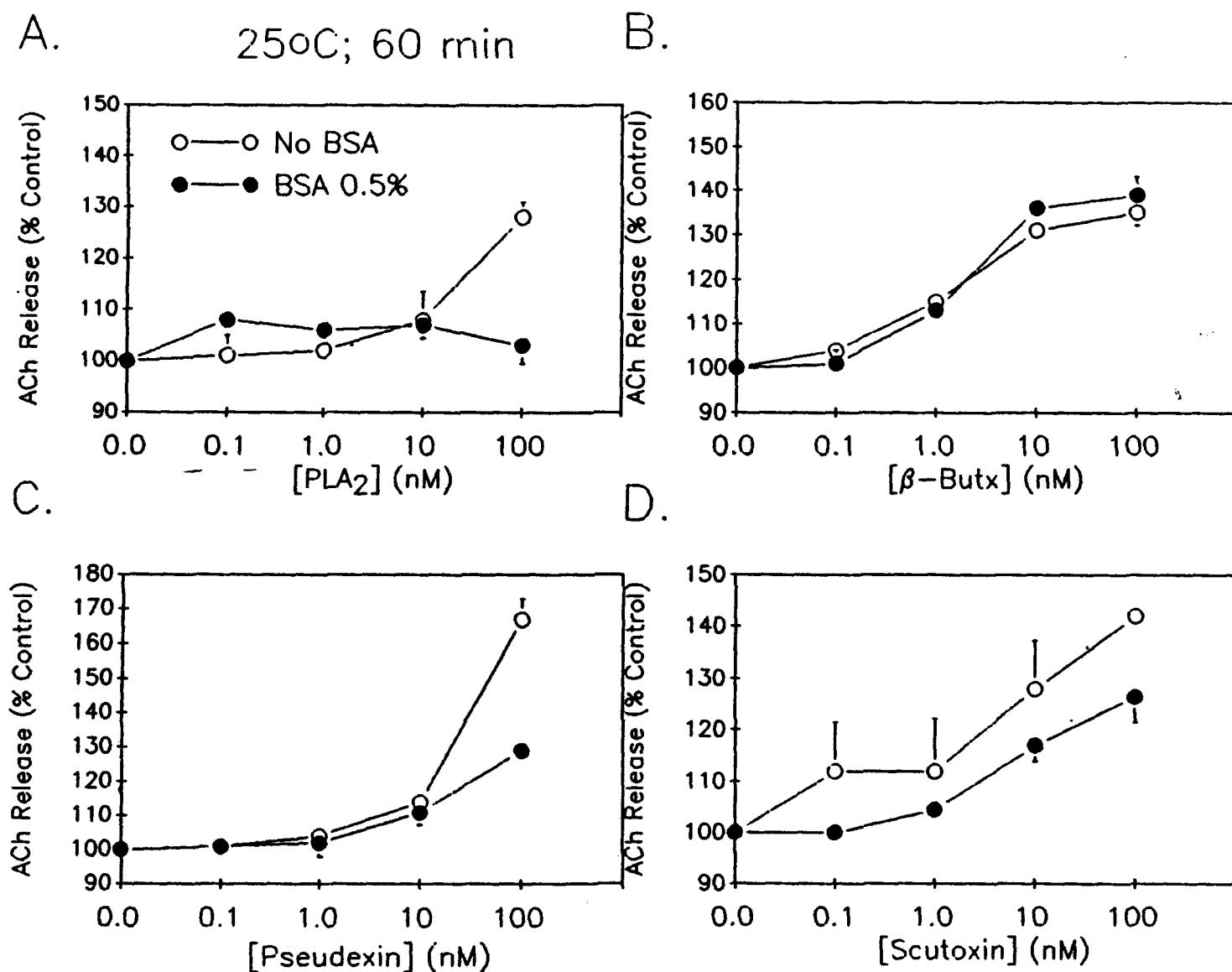
## FIGURE LEGENDS



**FIGURE 1.** Protocol for determining reversibility of antagonism of choline uptake by toxins. Synaptosomal preparations are either (A) incubated with toxin 1 min, washed and incubated with toxin for 59 more min, or (B) incubated with toxin for 1 min, washed and incubated without toxin for 59 min. Choline uptake is then determined at 37°C for 4 min.



**FIGURE 2.** Lack of reversibility of the inhibition of Ch uptake in synaptosomes by the toxins. The toxins were incubated with or without BSA as indicated at the bottom of the figure. The toxins examined were: β-Butx (bars 1,2,5,6); pseudexin (bars 3,4,7,8) and the *Naja naja atra* PLA<sub>2</sub> (bars 9,10). The synaptosomes were either incubated with toxin for 1 (bars 1,3,5,7,9) or 60 (bars 2,4,6,8,10) min, as described in Figure 2. Ch uptake was then determined (see Figure 1).



**FIGURE 3.** Effects of toxins on acetylcholine release from mouse brain synaptosomes. Synaptosomes were preloaded with [<sup>14</sup>C]choline (2 μM; 25°C; 30 min) and then incubated (± toxin) in the absence (open symbols) or presence (filled symbols) of BSA (0.5%) for 60 min 25°C. The synaptosomal suspensions were centrifuged and the radioactivity in the supernatant associated with ACh selectively extracted and quantitated by liquid scintillation counting. Each data point is comprised of 3-6 determinations and includes the SD bar.

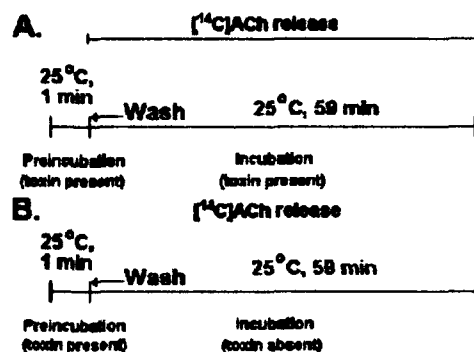


FIGURE 4. Protocol for determining reversibility of stimulation of ACh release by toxins. Synaptosomal preparations are either (A) incubated with toxin 1 min, washed and incubated with toxin for 59 more min, or (B) incubated with toxin for 1 min, washed and incubated without toxin for 59 min. ACh release is collected over the 59 min incubation period.

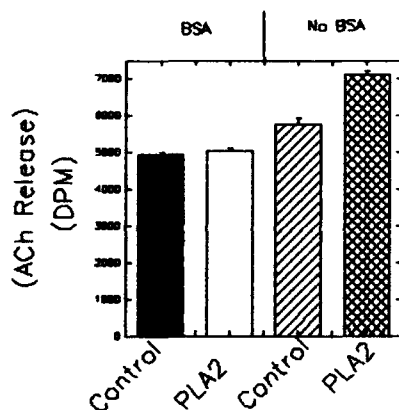


FIGURE 5. Effects of BSA on the stimulation of ACh release by the *Naja naja kaouthia* PLA<sub>2</sub> in the presence and absence of BSA. The mean  $\pm$  SD (3) is shown for a synaptosomal preparation incubated with 100 nM toxin.

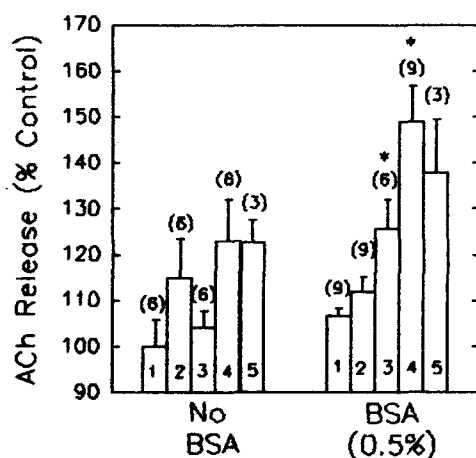


FIGURE 6. Comparison of ACh release with or without BSA 0.5% after 5 min. ACh was determined during incubation with 100 nM of the following toxins for 5 or 60 min: (1) *N.n.a.* PLA<sub>2</sub>; (2) *N.n.k.* PLA<sub>2</sub>; (3)  $\beta$ -Butx; (4) scutoxin; (5) pseudexin B. Neostigmine (100  $\mu\text{M}$ ) was present for the entire incubation and ACh collection. Mean  $\pm$  SD for the indicated number of determinations. Asterisk denotes a significant ( $P < 0.0001$ ) difference from no BSA by two-tailed grouped t-test.

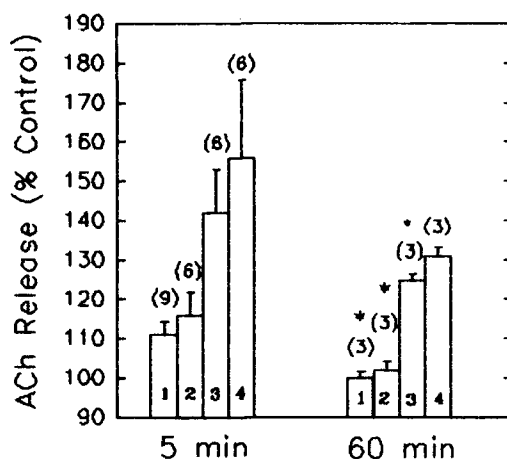


FIGURE 7. Comparison of ACh release in 1% BSA after 5 and 60 min. ACh was determined during incubation with 100 nM of the following toxins for 5 or 60 min: (1) *N.n.a.* PLA<sub>2</sub>; (2) *N.n.k.* PLA<sub>2</sub>; (3)  $\beta$ -Butx; (4) scutoxin. Neostigmine (100  $\mu$ M) was present for the entire incubation and ACh collection. Mean  $\pm$  SD for the indicated number of determinations. Asterisk denotes a significant ( $P < 0.05$ ) difference from 5 min by two-tailed grouped t-test.

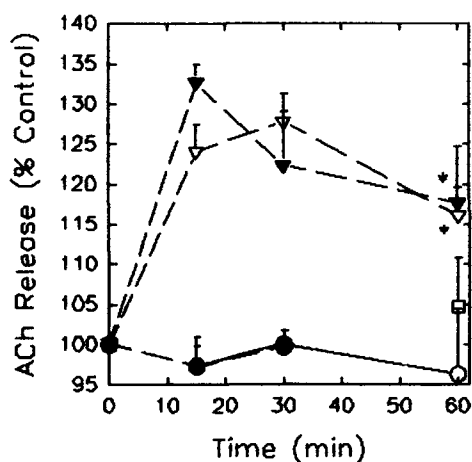


FIGURE 8. Time course of ACh release induced in the presence of BSA 0.5% by a 100 nM concentration of the following toxins: *Naja naja atra* PLA<sub>2</sub> (unfilled circles); *Naja naja kaouthia* PLA<sub>2</sub> (filled circles);  $\beta$ -Butx (unfilled inverted triangles); scutoxin (filled inverted triangles); pseudexin B (unfilled square). All values are mean  $\pm$  SD ( $n = 3$ ). Neostigmine (100  $\mu$ M) was present for the entire incubation and ACh collection. Asterisks denote a value different ( $P < 0.05$ ) from the 15 min point when all times were compared by a one-way ANOVA and Shefe test.

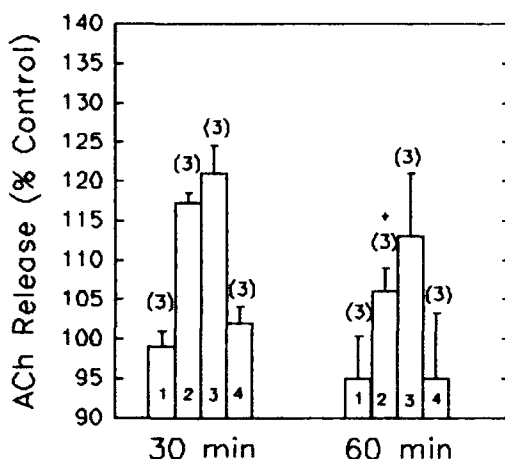


FIGURE 9. Comparison of ACh release in 0.5% BSA after 30 and 60 min. ACh was determined during incubation with 100 nM of the following toxins for 30 or 60 min in the absence of neostigmine: (1) *N.n.a.* PLA<sub>2</sub>; (2)  $\beta$ -Butx; (3) scutoxin; (4) pseudexin B. Following incubation with toxin, neostigmine (100  $\mu$ M) was added for a 4 min period. Mean  $\pm$  SD for the indicated number of determinations. Asterisk denotes a significant ( $P < 0.01$ ) difference from 30 min by two-tailed grouped t-test.

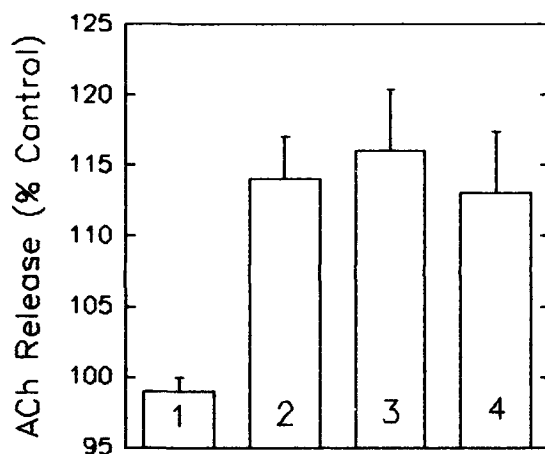


FIGURE 10. Effects of neostigmine on the stimulation of ACh release by  $\beta$ -Butx (100 nM). Preparations were incubated for 60 min without (bar 1) or with (bars 2-4) toxin and then incubated without (bar 1) or with toxin (bars 2-4) for 4 min with 0  $\mu$ M (bar 2), 100  $\mu$ M (bar 3), or 400  $\mu$ M (bars 1 and 4) of neostigmine. The ACh release was compared to controls in which neostigmine was not added for the 4 min incubation period. All values are mean  $\pm$  SD ( $n = 3$ ).

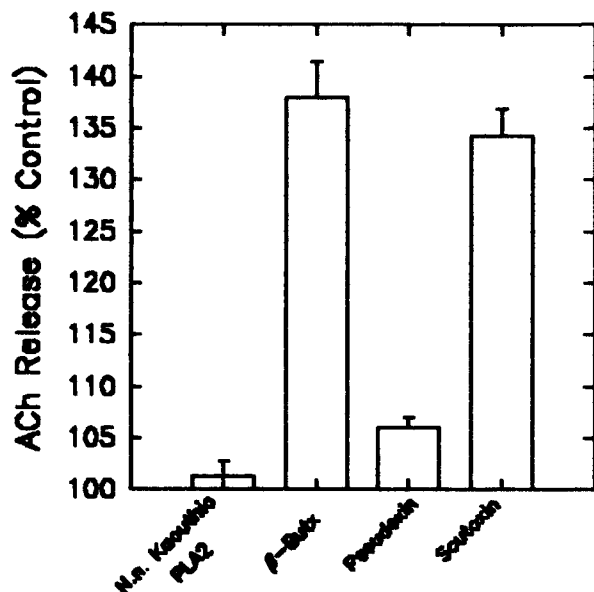


FIGURE 11. Comparisons of the effects on ACh release from **RAT BRAIN SYNAPTOSOMES** between three PSNTXs and a nonPSNTX PLA<sub>2</sub>. Rat brain synaptosomes were isolated as previously reported for mouse brain synaptosomes. Synaptosomes were incubated with the toxins (100 nM) at 25°C for 60 min in buffer containing BSA 0.5% and neostigmine (200 μM) and the total ACh release over this period determined. Values are the mean  $\pm$  SD for three determinations.

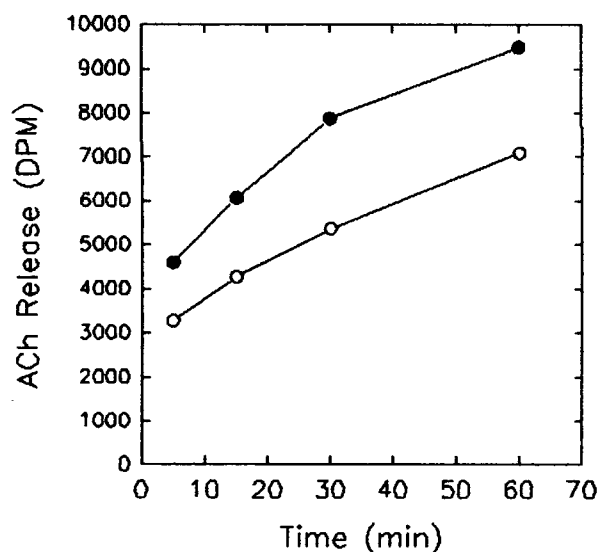


FIGURE 12. Time course for effects on ACh release from **RAT BRAIN SYNAPTOSOMES** of β-Butx. Rat brain synaptosomes were isolated as previously reported for mouse brain synaptosomes. Synaptosomes were incubated with (●) or without (○) the toxin (100 nM) at 25°C for the indicated time in buffer containing BSA 0.5% and neostigmine (200 μM) and the total ACh release over this period determined. Values are the mean of duplicate determinations.



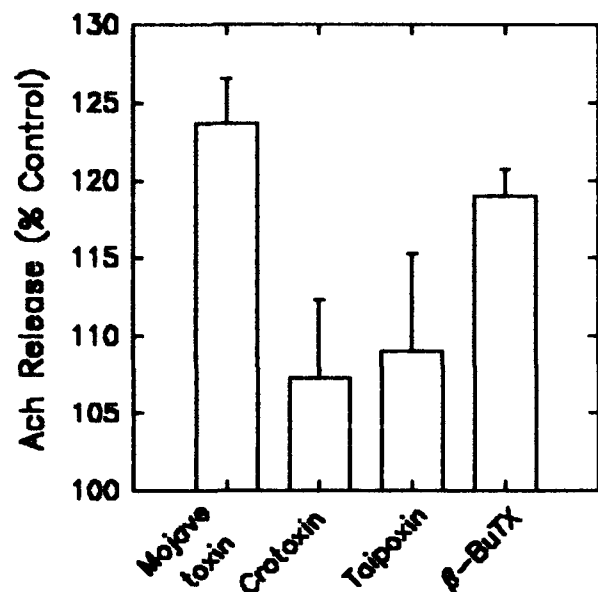


FIGURE 13. Effects of PSNTXs on ACh release from **RAT BRAIN** synaptosomes. Rat brain synaptosomes were isolated as previously reported for mouse brain synaptosomes. Synaptosomes were incubated with the toxins (100 nM) at 25°C for 5 min in buffer containing BSA 0.5% and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Values are the mean  $\pm$  SD for three determinations.

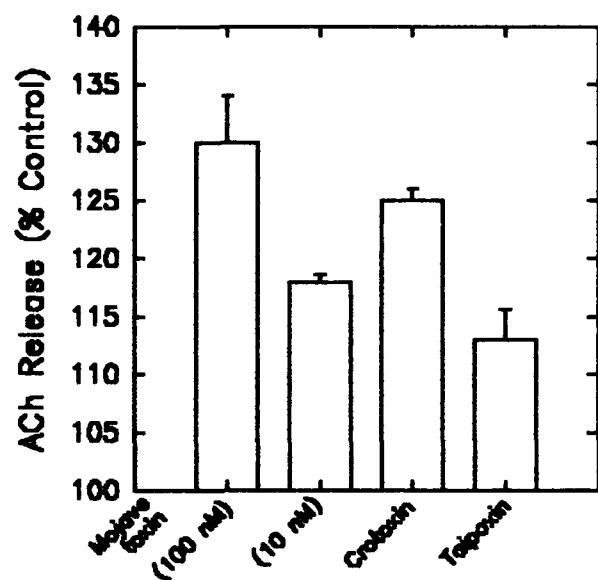


FIGURE 14. Effects of PSNTXs on ACh release from **RAT BRAIN** synaptosomes. Rat brain synaptosomes were isolated as previously reported for mouse brain synaptosomes. Synaptosomes were incubated with the toxins (100 nM unless otherwise indicated) at 25°C for 5 min in buffer containing BSA 0.5% and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Values are the mean  $\pm$  SD for three determinations.

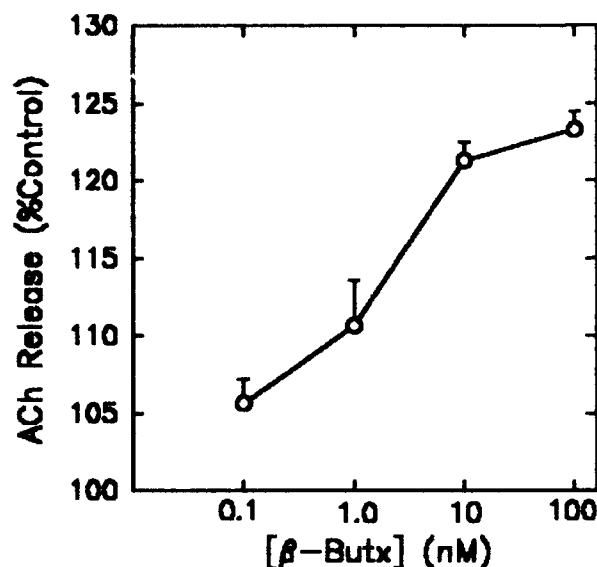


FIGURE 15. Dose-response for  $\beta$ -Butx effect on ACh release in **RAT BRAIN** synaptosomes. Rat brain synaptosomes were isolated as previously reported for mouse brain synaptosomes. Synaptosomes were incubated with the toxin at 25°C for 5 min in buffer containing BSA 0.5% and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Values are the mean  $\pm$  SD for three determinations.

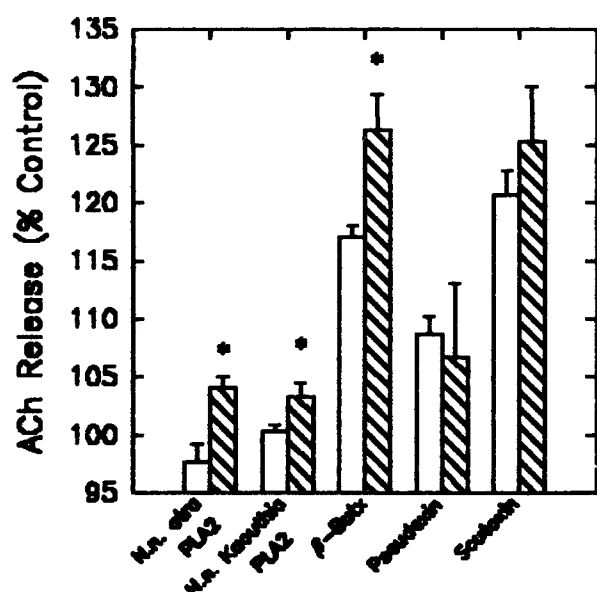


FIGURE 16. Effects of incubation with 0.5% BSA (open bars) compared to 1.0% (diagonal bars) BSA on toxin-induced ACh release in mouse brain synaptosomes. Synaptosomes were incubated with the toxins (100 nM) at 25°C for 60 min in buffer containing BSA (0.5% or 1%) and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Values are the mean  $\pm$  SD for three determinations, except for the *N.n. kaouthia* PLA<sub>2</sub> and pseudexin toxins in 1% BSA in which there were six determinations. Asterisk indicates greater ( $P < 0.05$ ) than absence of BSA.

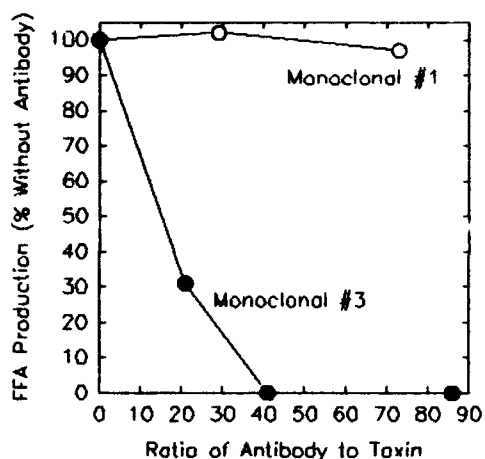


FIGURE 17. Effects of two monoclonal antibodies prepared toward a mixture of pseudexins (A, B, C) on the  $PLA_2$  activity of pseudexin B, as determined on triton X-100:egg yolk PC (2:1) mixed micelles. The toxin was pretreated with the indicated antibody ratio for 60 min ( $25^\circ\text{C}$ ).  $PLA_2$  activity ( $0.3 \mu\text{g enzyme/ml}$ ) was then determined for 30 min at  $37^\circ\text{C}$ . Values are the mean of duplicate determinations.

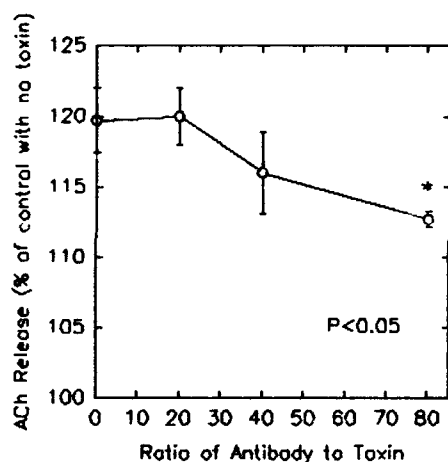


FIGURE 18. Effects of monoclonal antibody #3 on ACh release from synaptosomes by pseudexin B ( $100 \text{ nM}$ ). Toxins were pretreated with the indicated antibody ratio for 120 min ( $25^\circ\text{C}$ ). Synaptosomes were incubated with or without pseudexin B for 30 min at  $25^\circ\text{C}$  in the absence of BSA. The percent stimulation of ACh release by pseudexin-treated synaptosomes relative to nontoxin treated preparations is indicated at each antibody concentration. Values are mean  $\pm$  SD ( $n=3$ ).

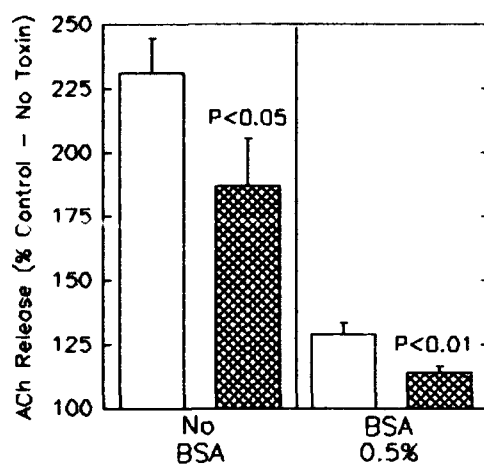


FIGURE 19. Effects of monoclonal antibody #3 on the stimulation of ACh release by scutoxin (100 nM) in the absence and presence of BSA 0.5%. Scutoxin was pretreated with antibody at a ratio of 80:1 (Ab:toxin) for 2 hrs at 25°C. The percent stimulation of ACh release by scutoxin-treated synaptosomes (30 min at 25°C) relative to nontoxin treated preparations is indicated in the absence (open bars) or presence (cross-hatched bars) of antibody. ACh release was determined in the absence (left panel) or presence (right panel) of BSA. Values are mean  $\pm$  SD (n=3).

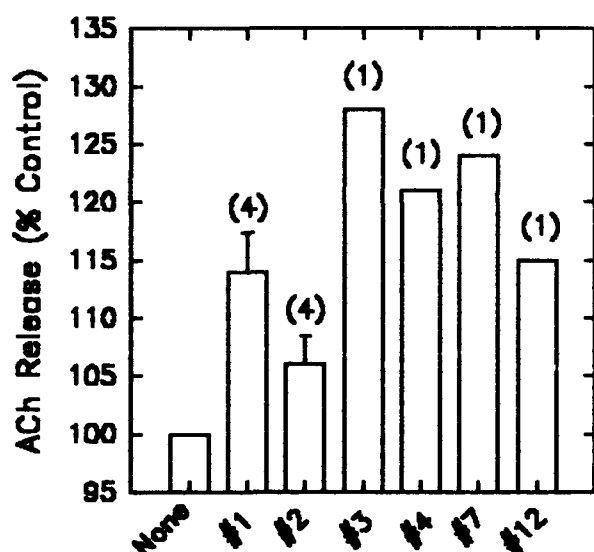
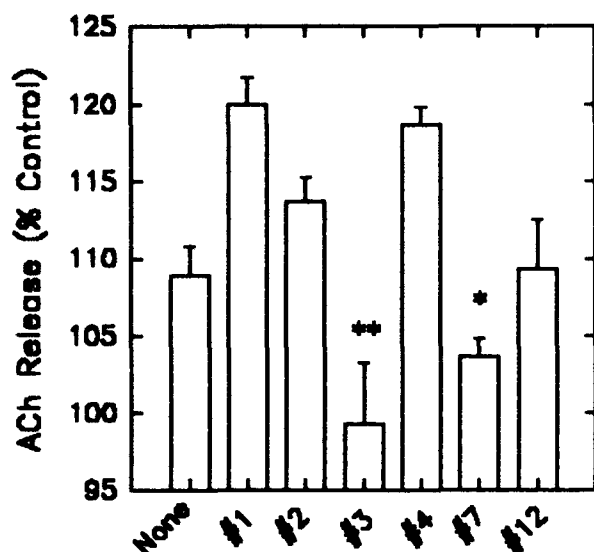
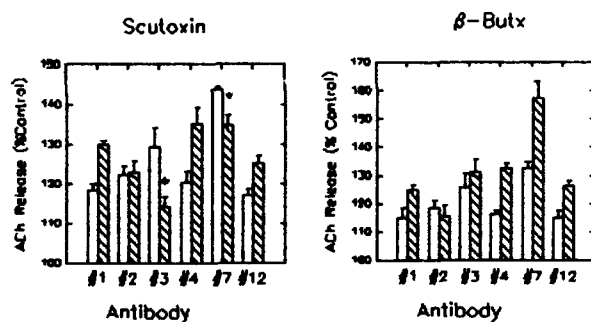


FIGURE 20. Effects of antibodies to pseudexin on ACh release in the absence of toxin in mouse brain synaptosomes. Synaptosomes were incubated with the antibodies (240  $\mu$ g) at 25°C for 30 min in buffer containing BSA (0.5%) and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Values are the mean  $\pm$  SD or mean for the number of determinations indicated in parentheses. A control for each experiment was normalized to 100% (mean of three determinations).



**FIGURE 21.** Effects of antibodies to pseudoxin on pseudoxin-induced ACh release in mouse brain synaptosomes. Synaptosomes were incubated with pseudoxin (100 nM; 3  $\mu$ g)  $\pm$  antibody (240  $\mu$ g) at 25°C for 30 min in buffer containing BSA (0.5%) and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Values are the mean  $\pm$  SD for three determinations, except for pseudoxin alone (n = 9), and antibody #3 (n = 6). Asterisk indicates less ( $P < 0.05$ ) than pseudoxin alone.



**FIGURE 22.** Cross reactivity of pseudoxin antibodies to ACh release induced by scut toxin and  $\beta$ -Butx in mouse brain synaptosomes. Synaptosomes were incubated with toxin (100 nM; 3  $\mu$ g) without (open bars) or with (diagonal bars) antibody (240  $\mu$ g) at 25°C for 30 min in buffer containing BSA (0.5%) and neostigmine (200  $\mu$ M) and the total ACh release over this period determined. Since the stimulation of ACh release exhibited some day-to-day variation,

separate controls (toxin alone) are shown for each antibody study. Values are the mean  $\pm$  SD for three determinations. Asterisk indicates less ( $P < 0.05$ ) than toxin alone.

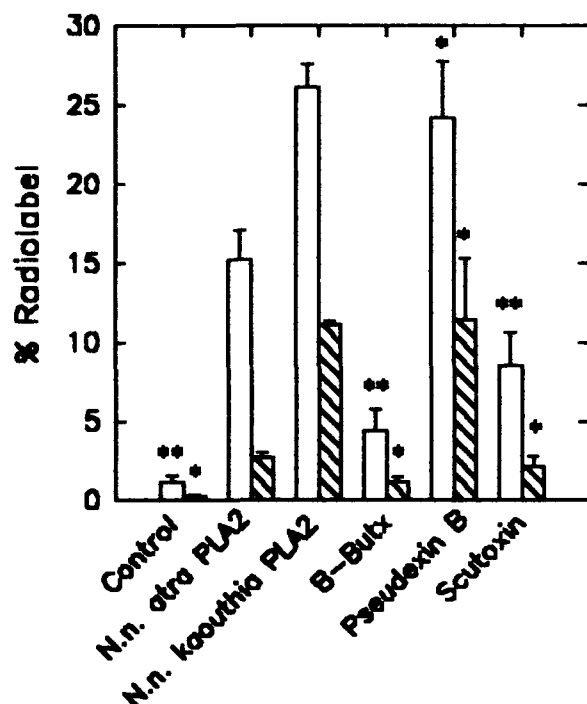


FIGURE 23A. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on free fatty acids (open bars) and LPC (diagonal bars) in differentiated NB41A3 cells (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:2 for an additional 3 days (Na<sup>+</sup> butyrate still present). Values (mean  $\pm$  SD; n = 3) for free fatty acids are expressed as percent total radiolabeled lipid and those for LPC are expressed as percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test; P < 0.05) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

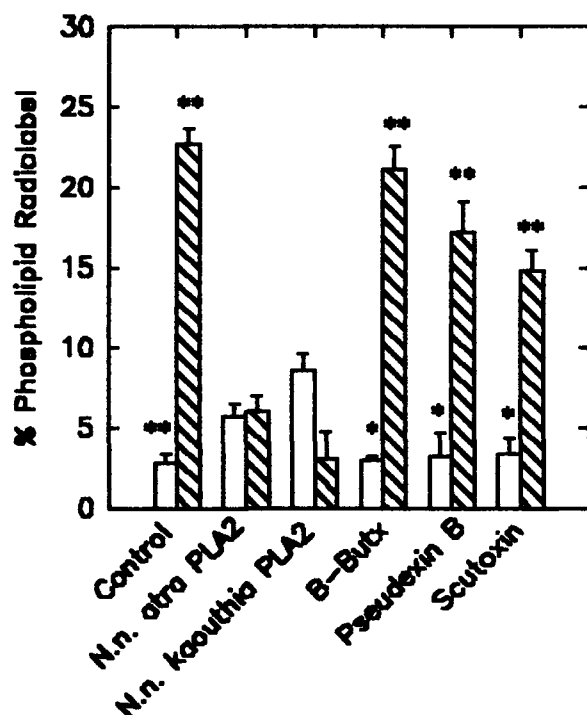


FIGURE 23B. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PS (open bars) and PE (diagonal bars) in differentiated NB41A3 cells (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:2 for an additional 3 days (Na<sup>+</sup> butyrate still present). Values are the mean  $\pm$  SD (n = 3) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test; P < 0.05) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s. Note: only the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s were different from control values for PS.

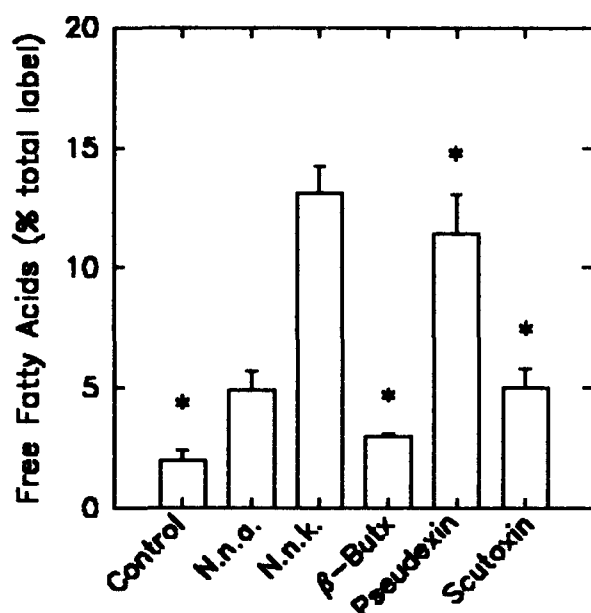


FIGURE 24A. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on free fatty acids in differentiated NB41A3 cells (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:0 for an additional 3 days (Na<sup>+</sup> butyrate still present). Values (mean  $\pm$  SD; n = 3) for are expressed as percent total radiolabeled lipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test;  $P < 0.05$ ) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

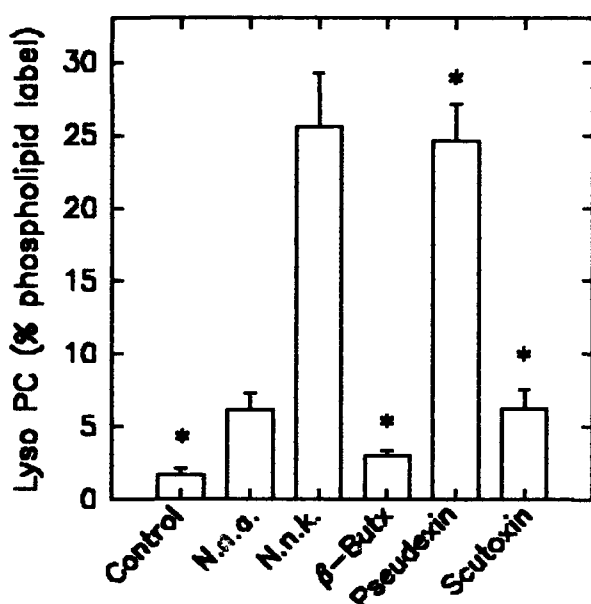


FIGURE 24B. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PC (LPC) in differentiated NB41A3 cells (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:0 for an additional 3 days (Na<sup>+</sup> butyrate still present). Values are the mean  $\pm$  SD (n = 3) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test;  $P < 0.05$ ) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

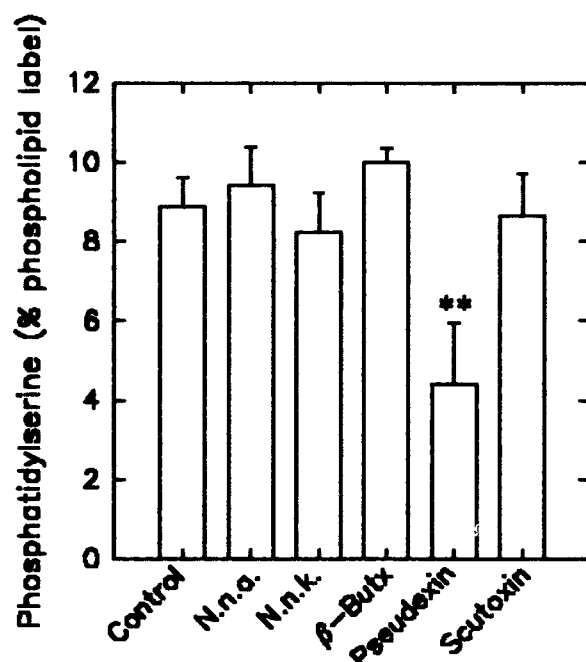


FIGURE 24C. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PS in differentiated NB41A3 cells (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:0 for an additional 3 days (Na<sup>+</sup> butyrate still present). Values are the mean  $\pm$  SD ( $n = 3$ ) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test;  $P < 0.05$ ) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

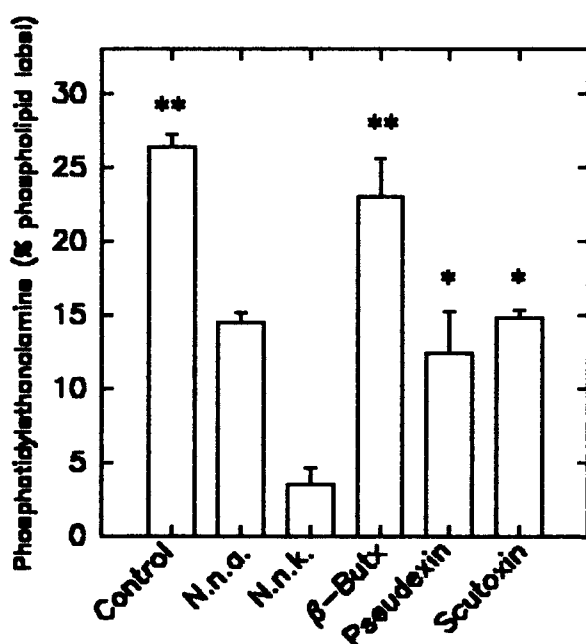


FIGURE 24D. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PE in differentiated NB41A3 cells (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:0 for an additional 3 days (Na<sup>+</sup> butyrate still present). Values are the mean  $\pm$  SD ( $n = 3$ ) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test;  $P < 0.05$ ) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.



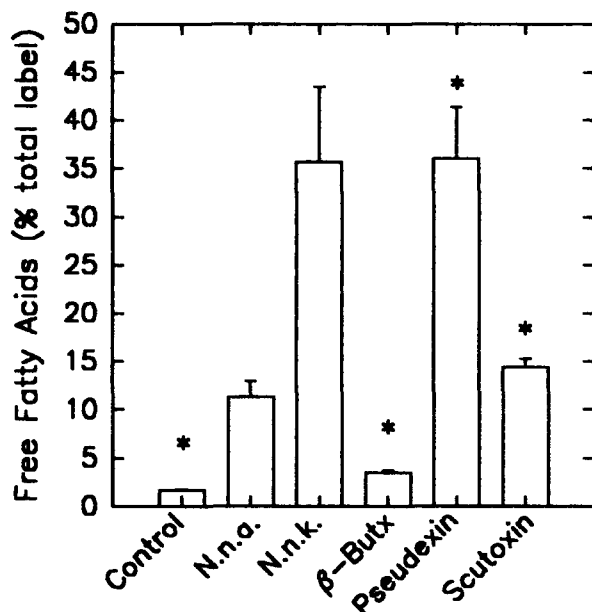


FIGURE 25A. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on free fatty acids in **NONDIFFERENTIATED** NB41A3 cells incubated 4 days (no Na<sup>+</sup> butyrate) radiolabeled with 18:2 for an additional 3 days. Values are the mean  $\pm$  SD ( $n = 3$ ) for percent of total radiolabeled lipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test;  $P < 0.05$ ) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

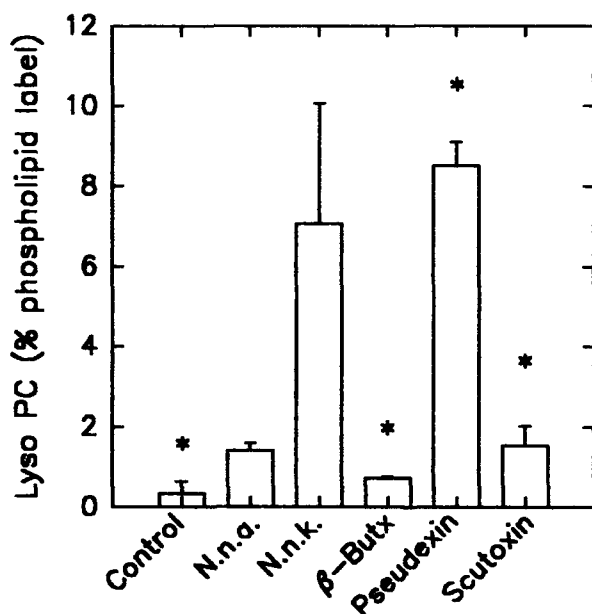


FIGURE 25B. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PC (LPC) in **NONDIFFERENTIATED** NB41A3 cells incubated 4 days (no Na<sup>+</sup> butyrate) radiolabeled with 18:2 for an additional 3 days. Values are the mean  $\pm$  SD ( $n = 3$ ) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test;  $P < 0.05$ ) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

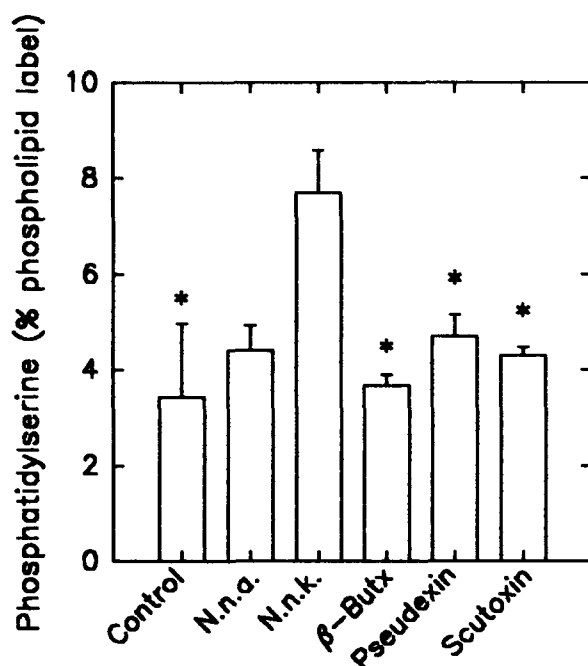


FIGURE 25C. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PS in **NONDIFFERENTIATED NB41A3** cells incubated 4 days (no Na<sup>+</sup> butyrate) radiolabeled with 18:2 for an additional 3 days. Values are the mean  $\pm$  SD (n = 3) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test; P<0.05) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s.

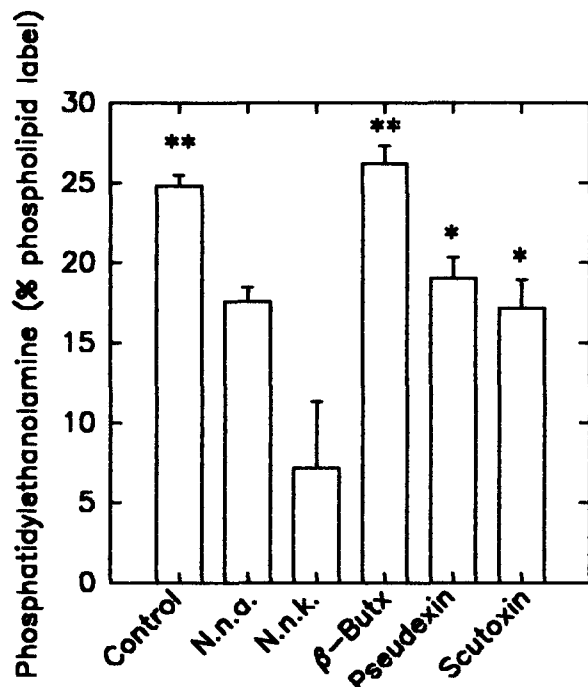
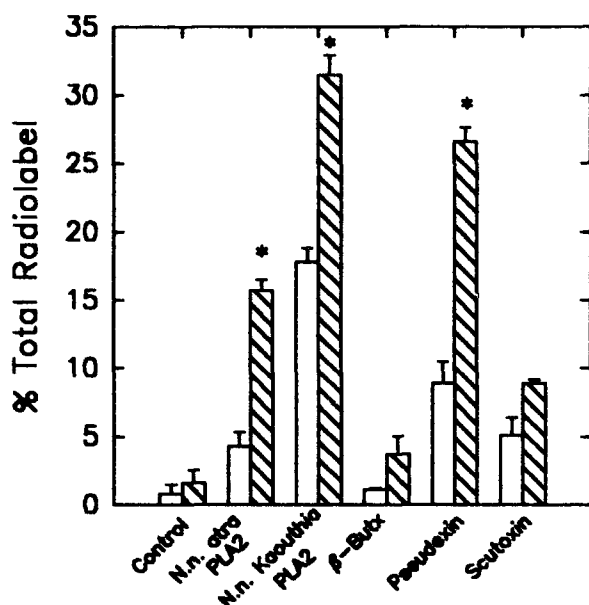
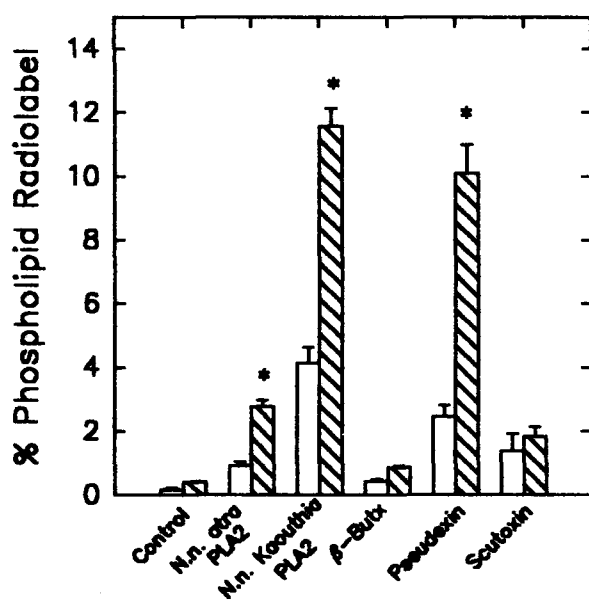


FIGURE 25D. Effects of five phospholipases A<sub>2</sub> (10 nM; 10 min; 37°C; 0.5% BSA) on PE in **NONDIFFERENTIATED NB41A3** cells incubated 4 days (no Na<sup>+</sup> butyrate) radiolabeled with 18:2 for an additional 3 days. Values are the mean  $\pm$  SD (n = 3) for percent radiolabeled phospholipid. The single asterisk denotes a value significantly different (ANOVA and Sheffe test; P<0.05) from either the *N.n. atra* or *N.n. Kaouthia* PLA<sub>2</sub>s. A double asterisks indicates a significant difference from both the *N.n. atra* and *N.n. Kaouthia* PLA<sub>2</sub>s. *Naja naja kaouthia* PLA<sub>2</sub>.



**FIGURE 26A.** Effects of BSA (0.5%) on free fatty acids produced by PLA<sub>2</sub>s and PSNTXs in a differentiated NB41A3 cell line. Cells were cultured in F-10 medium for four days (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:2 for an additional 3 days (Na<sup>+</sup> butyrate still present). Cells were washed with F-10 supplemented with Ca<sup>2+</sup> (2 mM) and incubated in this medium without (open bars) or with (diagonal lines) BSA for 10 min at 37°C with toxin (10 nM). Lipids were extracted and analyzed by 1-D TLC and a radioactivity scanner. Values are mean  $\pm$  SD (n = 3). Asterisks indicate value different in the presence than in the absence of BSA (ANOVA and Sheffe

test; P < 0.05).



**FIGURE 26B.** Effects of BSA (0.5%) on PC (LPC) produced by PLA<sub>2</sub>s and PSNTXs in a differentiated NB41A3 cell line. Cells were cultured in F-10 medium for four days (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:2 for an additional 3 days (Na<sup>+</sup> butyrate still present). Cells were washed with F-10 supplemented with Ca<sup>2+</sup> (2 mM) and incubated in this medium without (open bars) or with (diagonal lines) BSA for 10 min at 37°C with toxin (10 nM). Lipids were extracted and analyzed by 1-D TLC and a radioactivity scanner. Values are mean  $\pm$  SD (n = 3). Asterisks indicate value different in the presence than in the absence of BSA (ANOVA and Sheffe test; P < 0.05).

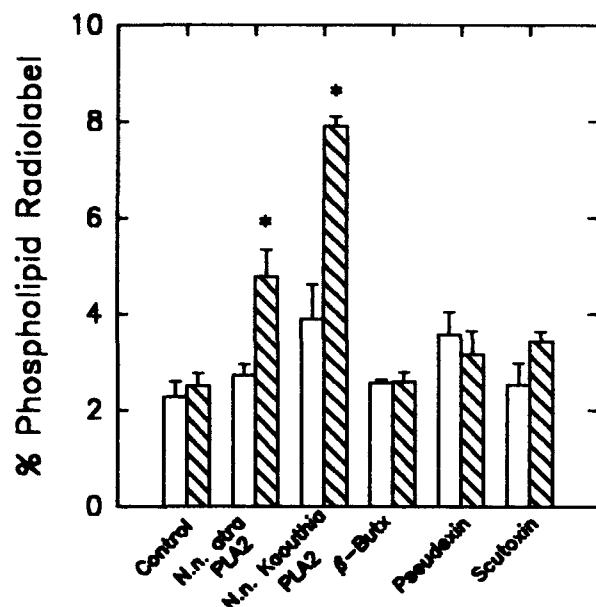


FIGURE 26C. Effects of BSA (0.5%) on phosphatidylserine produced by PLA<sub>2</sub>s and PSNTXs in a differentiated NB41A3 cell line. Cells were cultured in F-10 medium for four days (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:2 for an additional 3 days (Na<sup>+</sup> butyrate still present). Cells were washed with F-10 supplemented with Ca<sup>2+</sup> (2 mM) and incubated in this medium without (open bars) or with (diagonal lines) BSA for 10 min at 37°C with toxin (10 nM). Lipids were extracted and analyzed by 1-D TLC and a radioactivity scanner. Values are mean  $\pm$  SD (n = 3). Asterisks indicate value different in the presence than in the absence of BSA (ANOVA and Sheffe

test; P < 0.05).

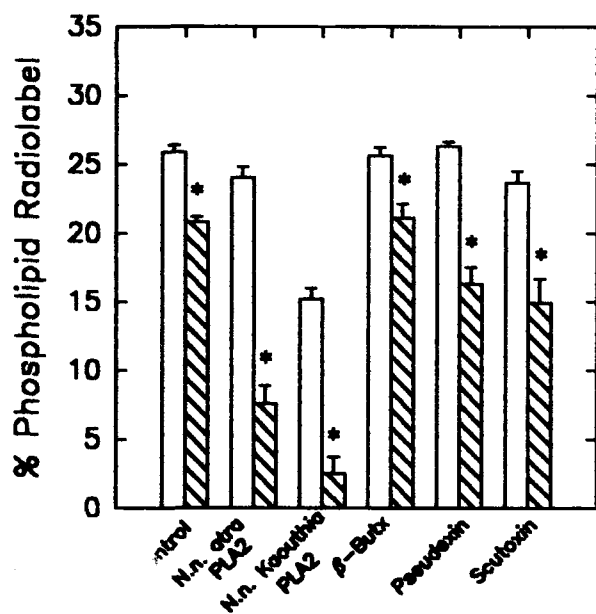
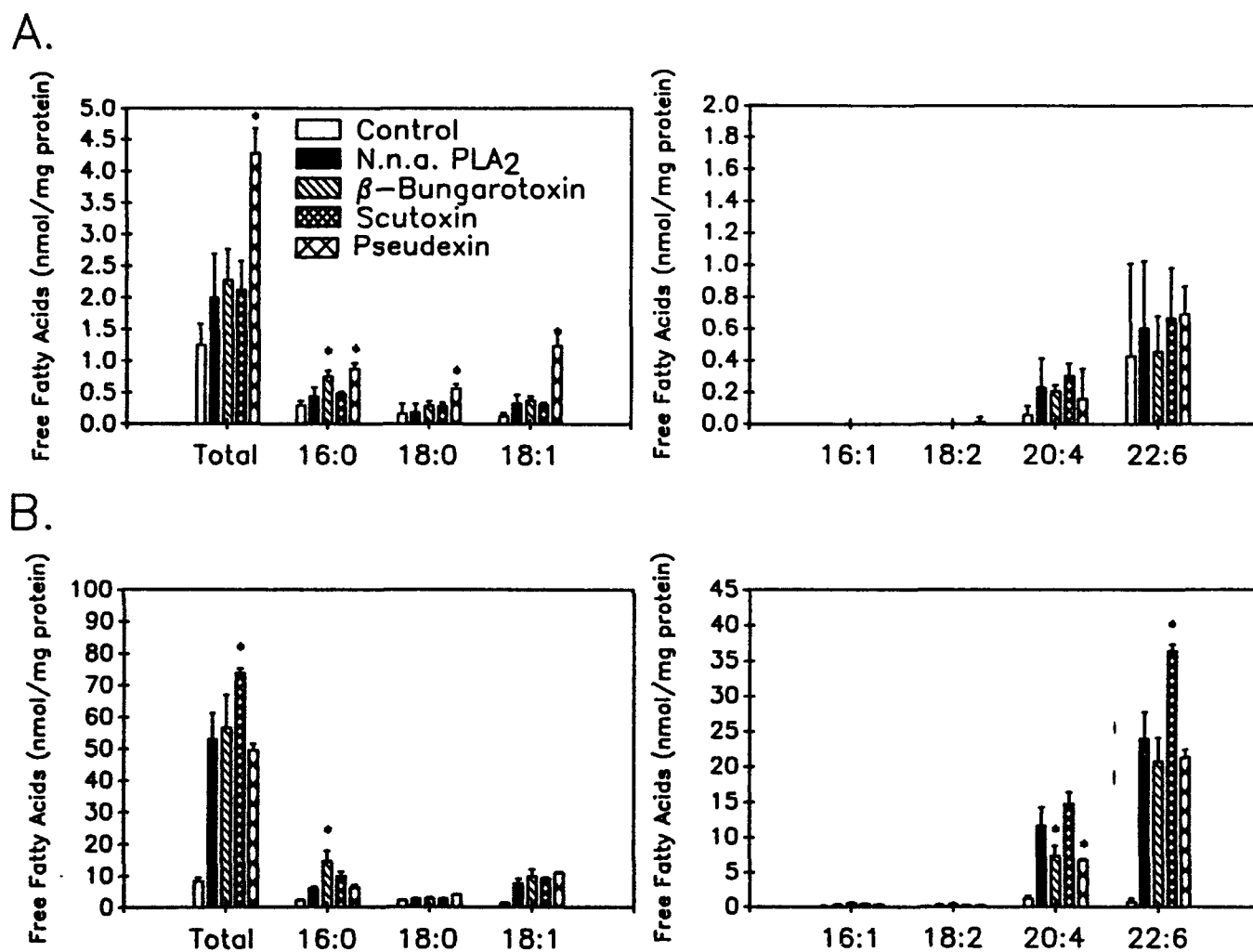
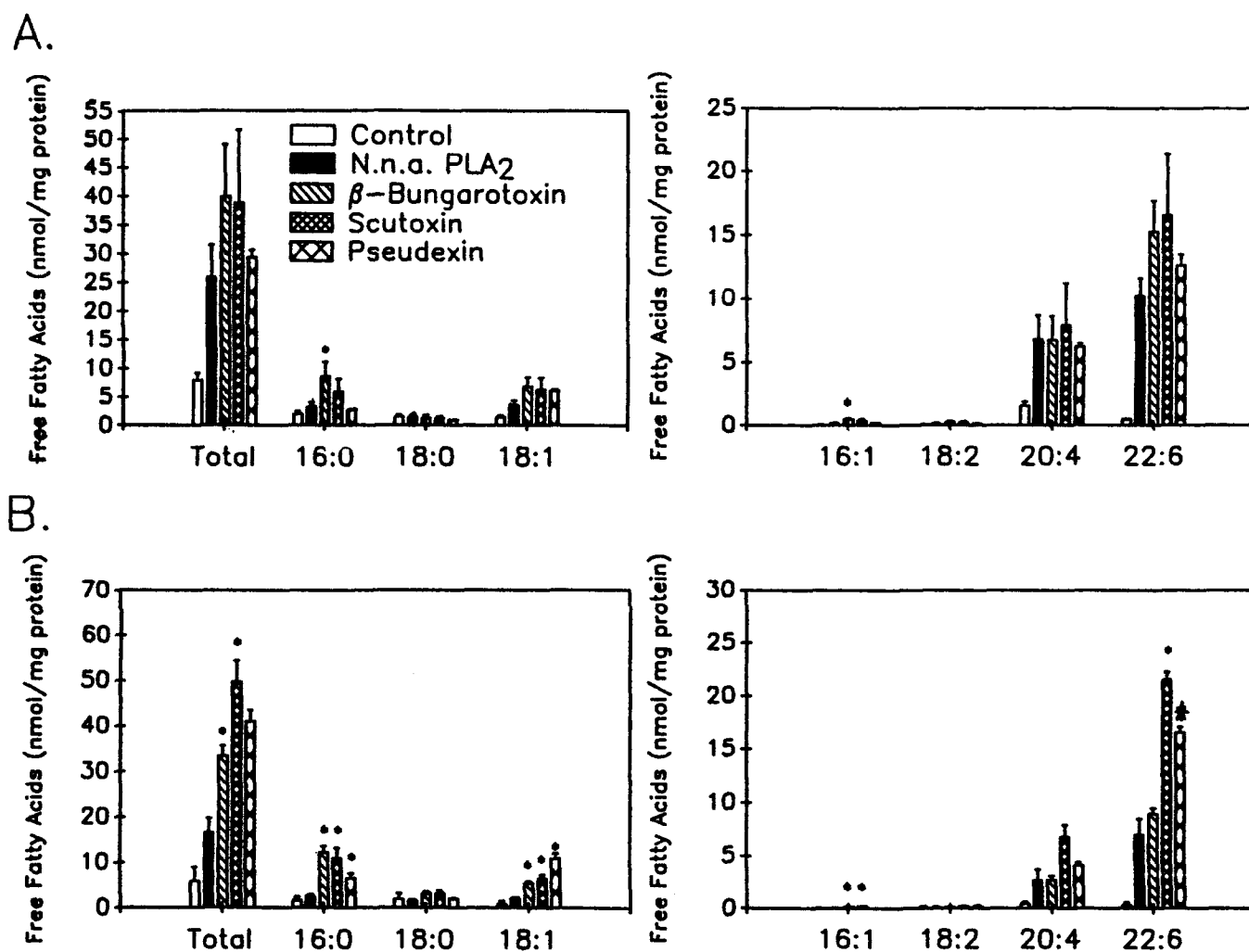


FIGURE 26D. Effects of BSA (0.5%) on phosphatidylethanolamine produced by PLA<sub>2</sub>s and PSNTXs in a differentiated NB41A3 cell line. Cells were cultured in F-10 medium for four days (plated on Day 1, Na<sup>+</sup> butyrate added on Day 2, incubated 3 days) radiolabeled with 18:2 for an additional 3 days (Na<sup>+</sup> butyrate still present). Cells were washed with F-10 supplemented with Ca<sup>2+</sup> (2 mM) and incubated in this medium without (open bars) or with (diagonal lines) BSA for 10 min at 37°C with toxin (10 nM). Lipids were extracted and analyzed by 1-D TLC and a radioactivity scanner. Values are mean  $\pm$  SD (n = 3). Asterisks indicate value different in the presence than in the absence of BSA (ANOVA and Sheffe test;

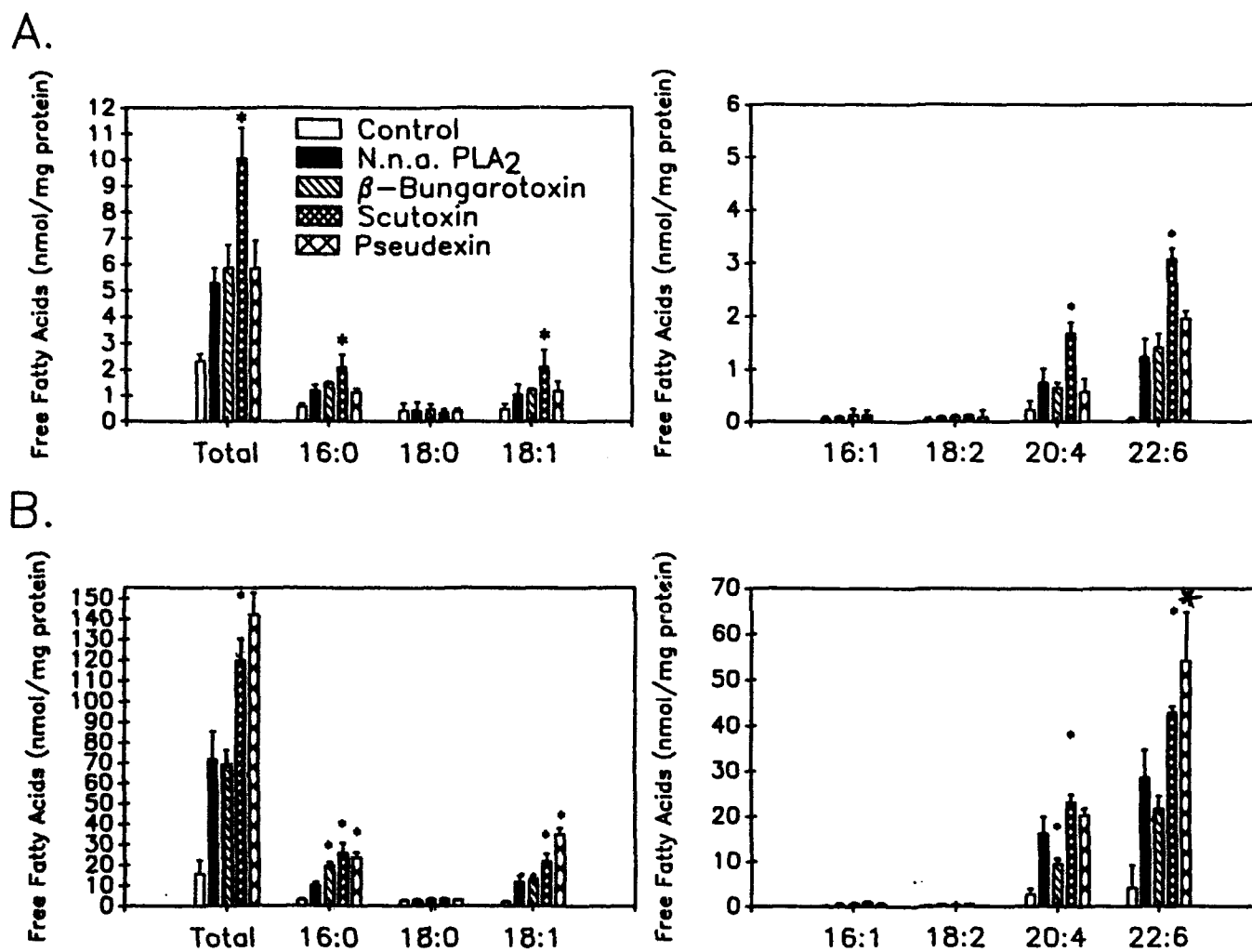
P < 0.05).



**FIGURE 27.** Analysis of FFAs (A) released to the incubation medium (supernatant) or (B) retained in mouse brain synaptosomes (pellet) during a 1 hr incubation at 25°C in the absence or presence of a 10 nM concentration of toxin. The mean and SD bars for four determinations are shown.

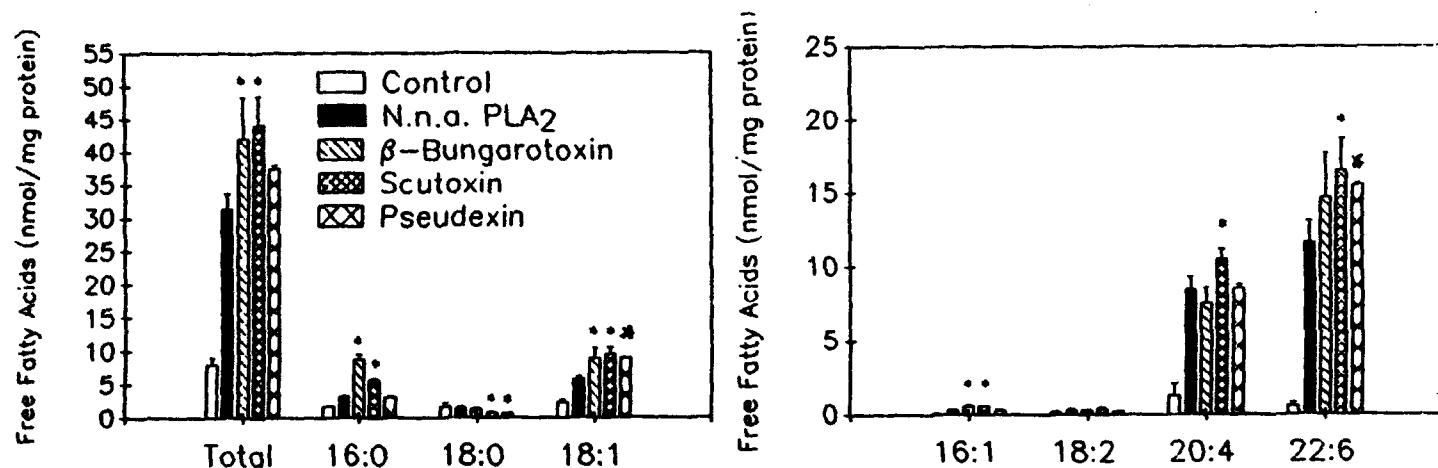


**FIGURE 28.** Extraction of FFAs by BSA (0.5%) in mouse brain synaptosomes incubated with PLA<sub>2</sub> toxins. FFAs were analyzed after a 1 hr incubation at 25°C in the absence or presence of a 10 nM concentration of toxin for those (A) released to the incubation medium (supernatant) or (B) retained in synaptosomes (pellet). The mean and SD bars for four determinations are shown.



**FIGURE 29.** Analysis of FFAs (A) released to the incubation medium (supernatant) or (B) retained in mouse brain synaptosomes (pellet) during a 1 hr incubation at 25°C in the absence or presence of a 100 nM concentration of toxin. The mean and SD bars for four determinations are shown.

A.



B.

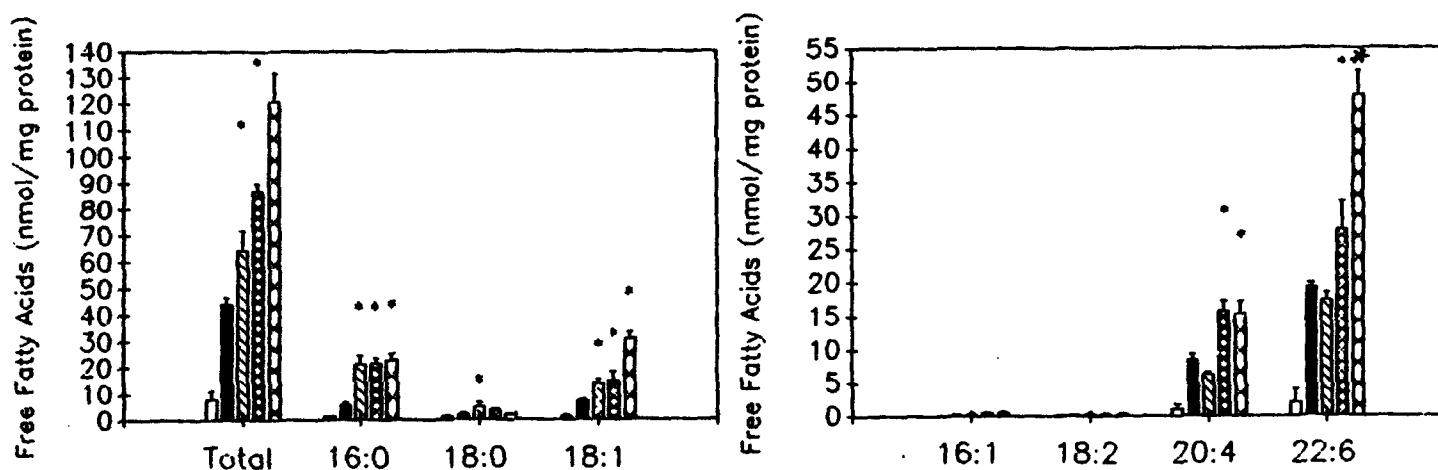


FIGURE 30. Extraction of FFAs by BSA (0.5%) in mouse brain synaptosomes incubated with PLA<sub>2</sub> toxins. FFAs were analyzed after a 1 hr incubation at 25°C in the absence or presence of a 100 nM concentration of toxin for those (A) released to the incubation medium (supernatant) or (B) retained in synaptosomes (pellet). The mean and SD bars for four determinations are shown.



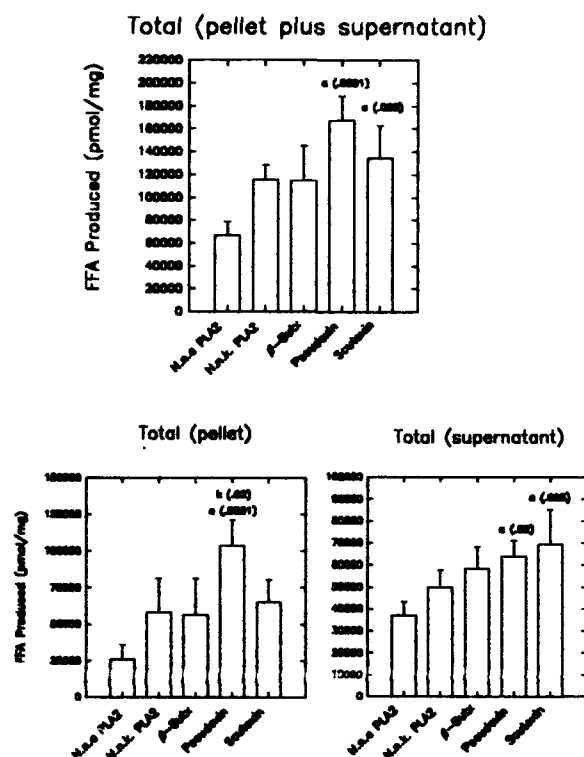
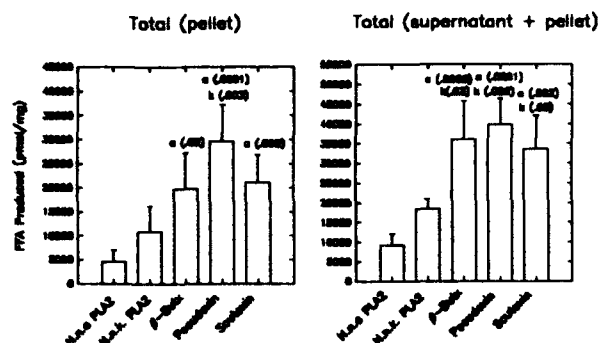


FIGURE 31A. Gas chromatographic analysis of total fatty acids liberated from synaptosomes by PLA<sub>2</sub>s and PSNTXs. Synaptosomes were incubated in buffer containing BSA 1% at 25°C for 60 min and toxin (100 nM). Preparations were centrifuged and the supernatant and pellet extracted separately for lipid analysis. Free fatty acid levels for control preparations were subtracted for the values in the figure. Values are the mean  $\pm$  SD for five preparations. Levels of significance (P-values) are provided for differences from the *Naja naja atra* (a) and *Naja naja kaouthia* (k) PLA<sub>2</sub>s, as determined by ANOVA and Sheffe test.



## Saturated (16:0 + 18:0)



ANOVA and Sheffe test.

FIGURE 31D. Gas chromatographic analysis of 16:0 and 18:0 fatty acids liberated from synaptosomes by PLA<sub>2</sub>s and PSNTXs. Synaptosomes were incubated in buffer containing BSA 1% at 25°C for 60 min and toxin (100 nM). Preparations were centrifuged and the supernatant and pellet extracted separately for lipid analysis. Free fatty acid levels for control preparations were subtracted for the values in the figure. Values are the mean  $\pm$  SD for five preparations. Levels of significance (P-values) are provided for differences from the *Naja naja atra* (a) and *Naja naja kaouthia* (k) PLA<sub>2</sub>s, as determined by

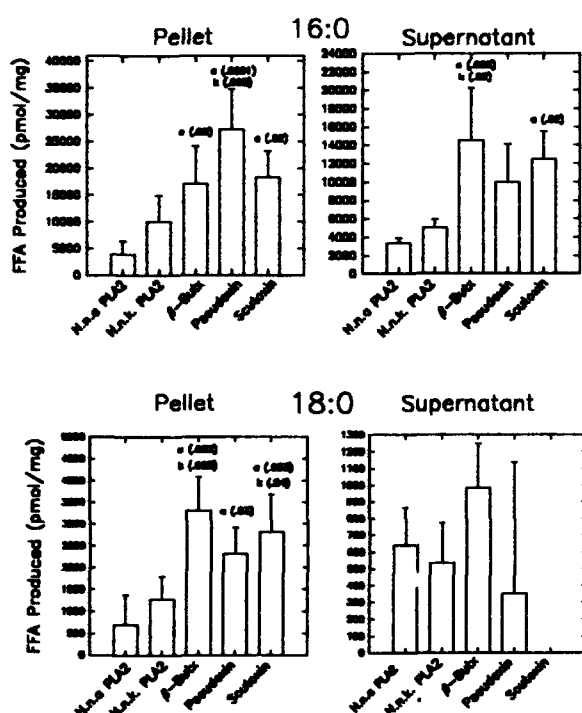
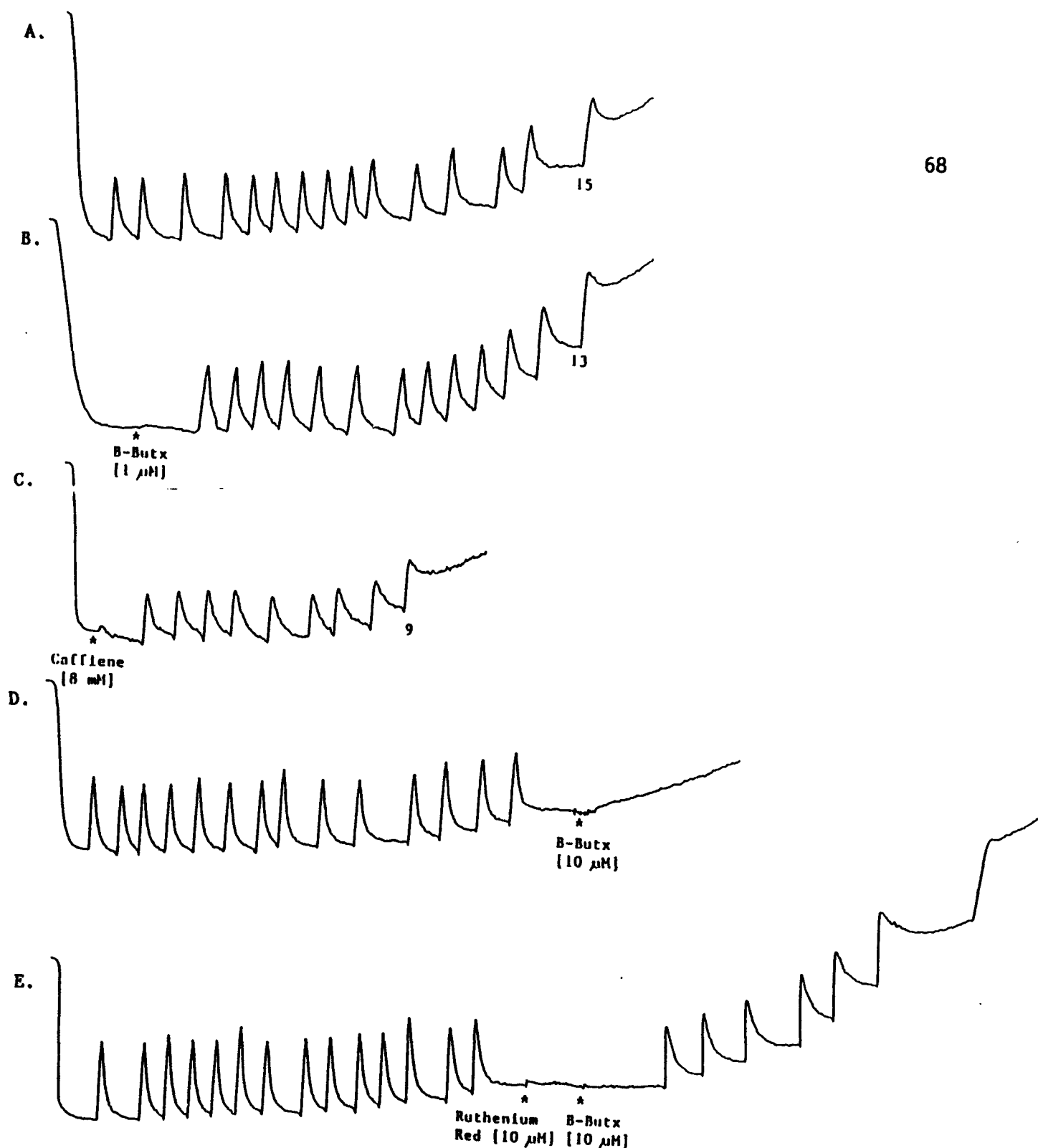


FIGURE 31E. Gas chromatographic analysis of 16:0 and 18:0 fatty acids liberated from synaptosomes by PLA<sub>2</sub>s and PSNTXs. Synaptosomes were incubated in buffer containing BSA 1% at 25°C for 60 min and toxin (100 nM). Preparations were centrifuged and the supernatant and pellet extracted separately for lipid analysis. Free fatty acid levels for control preparations were subtracted for the values in the figure. Values are the mean  $\pm$  SD for five preparations. Levels of significance (P-values) are provided for differences from the *Naja naja atra* (a) and *Naja naja kaouthia* (k) PLA<sub>2</sub>s, as determined by ANOVA and Sheffe test.



**FIGURE 32.** Effects of  $\beta$ -Butx on  $\text{Ca}^{2+}$  release. (A)  $\text{Ca}^{2+}$  was added in successive 1.5 nmol pulses to terminal cisternae-containing heavy sarcoplasmic reticulum fractions until the TCICR was reached; (B)  $\beta$ -Butx was added after ATP-stimulated  $\text{Ca}^{2+}$  uptake reduced the TCICR to 13 pulses or 87% of the control TCICR; (C) Effects of caffeine 8 mM on the TCICR; (D)  $\beta$ -Butx induces immediate  $\text{Ca}^{2+}$  release from terminal cisternae preloaded with  $\text{Ca}^{2+}$ ; (E) Ruthenium red (10  $\mu\text{M}$ ) antagonizes  $\beta$ -Butx-induced  $\text{Ca}^{2+}$  release.

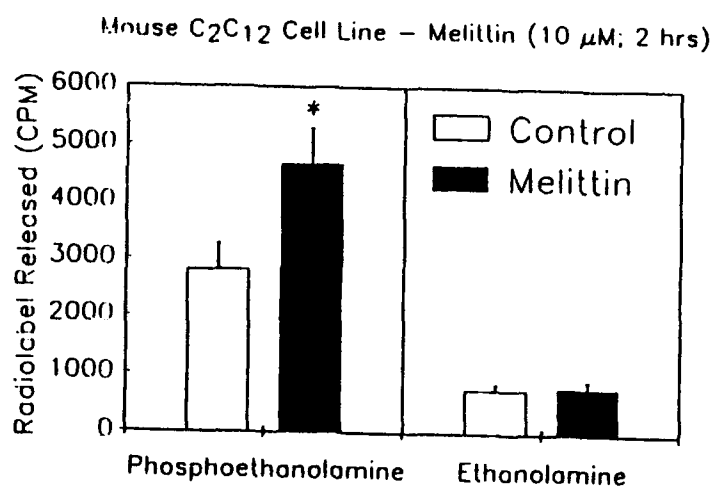


FIGURE 33. Phosphoethanolamine and ethanolamine release from mouse C<sub>2</sub>C<sub>12</sub> cell line treated 120 min with melittin (10  $\mu$ M). Cells were preradiolabeled with [<sup>14</sup>C]linoleic acid (10  $\mu$ M, 2 days) and with [<sup>14</sup>C]ethanolamine (10  $\mu$ M, 2 days) to radiolabel phosphatidylethanolamine.

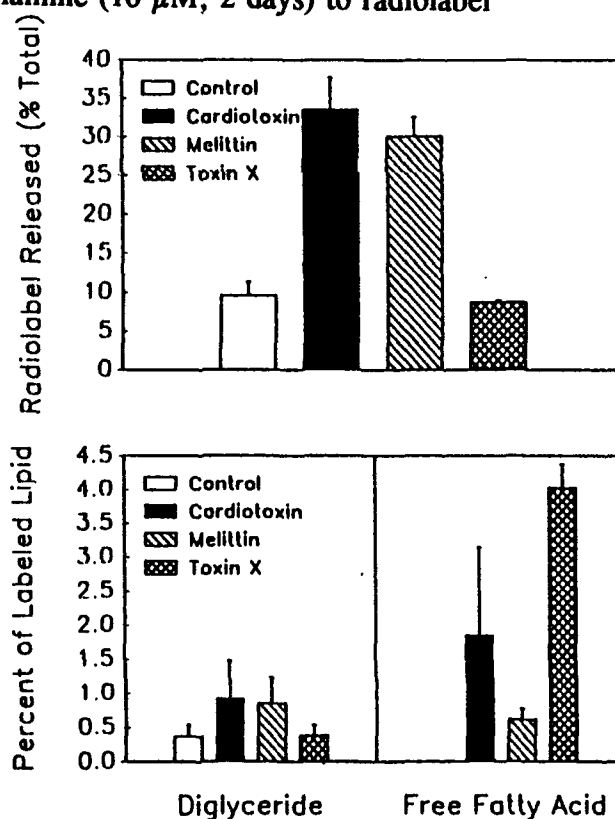


FIGURE 34. Comparison of the effects of cardiotoxin, melittin and Toxin X (all at 10  $\mu$ M concentrations) on radioactivity release (Upper Panel) into supernatant (expressed as % total radiolabel incorporated into the cells) from and on diglyceride and FFA production (Bottom Panel) in a mouse C<sub>2</sub>C<sub>12</sub> cell line. The cells were prelabeled with linoleic acid (10  $\mu$ M, 3 days) and incubated for 2 hrs with toxin.

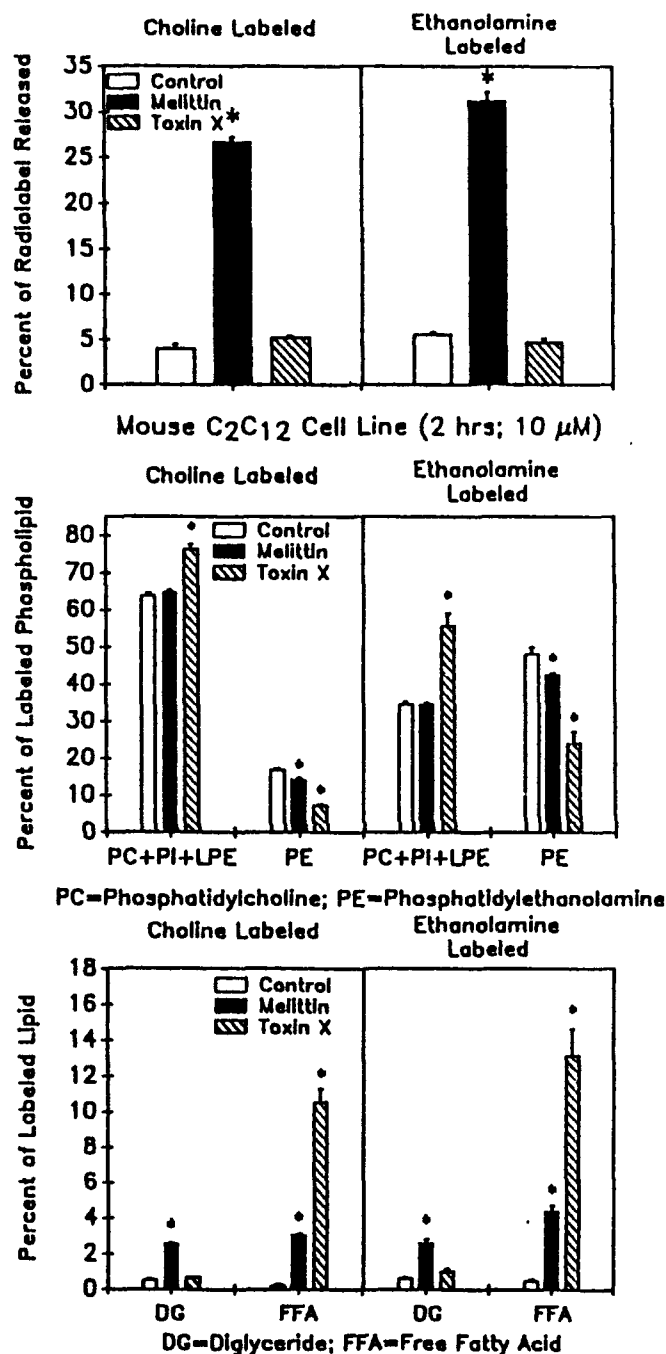
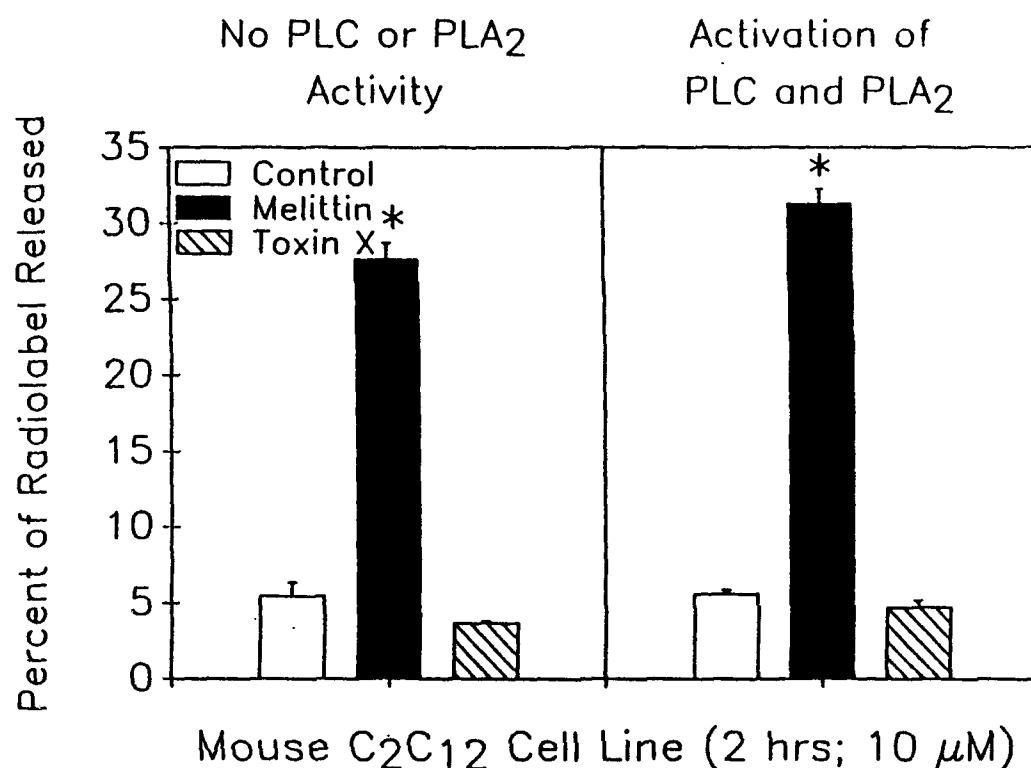


FIGURE 35. Comparison of the effects of melittin and Toxin X (at 10  $\mu$ M concentrations) on mouse C<sub>2</sub>C<sub>12</sub> cells preradiolabeled with either choline or ethanolamine. Upper Panel: radioactivity release into supernatant (expressed as % total radiolabel incorporated into the cells) from cells. Middle Panel: PC+PI+LPE and PE generated in cells. Bottom Panel: diglyceride and FFA production in cells. The cells were prelabeled with [<sup>14</sup>C]linoleic acid and either [<sup>14</sup>C]choline or [<sup>14</sup>C]ethanolamine (10  $\mu$ M of each, 3 days) incubated for 2 hrs with toxin.



**FIGURE 36.** Relationship between activation of  $PLA_2$  or PLC and release of radioactivity into the incubation medium. There was no activation of  $PLA_2$  or PLC in the cells in the Left Panel, as determined by a lack of DG or FFA production. Significant levels of DG and FFA were produced in the cultures in the Right Panel. In both cases the same amount of radioactivity was release into the incubation medium.

## CONCLUSIONS

### Effects of Presynaptic Neurotoxins on Ch Uptake and ACh Release in Synaptosomes

The PSNTXs irreversibly inhibit  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -independent choline uptake into synaptosomes, an action associated with the basic subunit for heterodimers, and this action is *not* completely inhibited by BSA. However, some antagonism may occur if the PSNTX has some degree of less specific action. For example, scutoxin has some myotoxic activity that may be due to nonPSNTX  $\text{PLA}_2$  activity in addition to its more specific neurotoxic action. In contrast, inhibition of choline uptake by nonPSNTX  $\text{PLA}_2$ s is completely inhibited by BSA.

The PSNTXs also irreversibly stimulate ACh release from mouse and rat brain synaptosomes and this action is *not* completely inhibited by BSA (although some antagonism may occur, as with choline uptake). This action has an early onset (ca. 5 min) and diminished with time (ca. > 15 min). The stimulation of ACh release by crotoxin, mojave toxin and taipoxin appears to be much more obvious in rat than in mouse brain synaptosomes. Increasing the BSA concentration from 0.5 to 1.0% increases the efficacy of the toxins in stimulating ACh release. Two antibodies to pseudexins A, B and C, effective in neutralizing pseudexin *in vivo* ( $\text{LD}_{50}$ ), also antagonized the stimulation of ACh release by pseudexin and scutoxin, but not  $\beta$ -Butx. Nonneutralizing antibodies had no effect on ACh release. In contrast to PSNTXs, nonPSNTX  $\text{PLA}_2$ s did not stimulate ACh release in the presence of BSA. A non $\text{PLA}_2$  peptide from *Trimeresurus wagleri* snake venom is also a PSNTX.

### Role of $\text{PLA}_2$ Activity in the Action of PSNTXs and CTXs

The PSNTXs are distinguished from the nonPSNTX  $\text{PLA}_2$ s in that the PSNTXs hydrolyze PS to the same extent as PE; whereas PS is not as good a substrate as PE for the nonPSNTX  $\text{PLA}_2$ s. The PSNTXs more readily hydrolyze differentiated over nondifferentiate nerve cells and less readily hydrolyze muscle culture phospholipids. In cell cultures the addition of BSA increases or has no effect on phospholipid hydrolysis; however, the substrate preference of the nonPSNTX  $\text{PLA}_2$ s increases for PE. In synaptosomes, there was no correlation between total FAs generated and presynaptic activity of the PSNTXs or non PSNTX  $\text{PLA}_2$ s. At a concentration of BSA of 0.5%, there was a consistent resistance to extraction from the synaptosomes of 16:0 and 18:1 produced by PSNTXs, suggesting that the PSNTXs were reaching a site inaccessible to the nonPSNTX  $\text{PLA}_2$ s and BSA. Increasing the BSA concentration to 1% revealed a greater overall hydrolysis of unsaturated FAs (16:0; 18:0) by the PSNTXs. Therefore, the PSNTXs have a very different spectrum of phospholipid hydrolysis from the nonPSNTX  $\text{PLA}_2$ s. These findings suggest a potential involvement of site-directed  $\text{PLA}_2$  activity in the action of the PSNTXs.

Even highly purified venom fractions of CTXs and melittin contain trace amounts of venom  $\text{PLA}_2$ . The  $\text{PLA}_2$  activity contaminating these fractions can be eliminated by either treating the fraction with *p*-BPB, or, in the case of melittin, by using synthetic toxin. While this contaminating  $\text{PLA}_2$  may enhance the toxicity of these toxins, it is not an activity that the peptides themselves possess.

### Action of CTXs, Melittin, Myotoxin $\alpha$ and $\beta$ -Butx on $\text{Ca}^{2+}$ Release

$\beta$ -Butx, CTX from *Naja naja kaouthia* venom, melittin and myotoxin  $\alpha$  all induce  $\text{Ca}^{2+}$



release from terminal cisternae preparations and this action is mediated through the ryanodine receptor ( $\text{Ca}^{2+}$  release channel). There is more species variability in the action of the CTX than melittin or myotoxin *a*. There is considerable interindividual variability within species for these three latter toxins. The effects on the channel appear to be very similar, yet slightly different for the same three toxins, especially based on the ryanodine binding studies.

#### Effects of the PSNTXs and CTXs on Tissue Lipid Metabolism

The PSNTXs do not activate tissue lipases in cell cultures. Snake venom CTXs and bee venom melittin activate tissue PLC activity (not tissue  $\text{PLA}_2$ ) within 1 min and the products of this activity continue to be generated for hours. The PLC activity is directed preferentially toward PE over PC. At high concentrations (10  $\mu\text{M}$ ) melittin also activates triglyceride lipolysis. Both CTX and melittin release cell membrane fragments into the incubation medium. The release of membrane fragments into the incubation medium and activation of PLC by these toxins is mostly independent of extracellular  $\text{Ca}^{2+}$  and is not antagonized by pertussis or cholera toxin. A fraction derived from *Crotalus viridis viridis*, initially mistaken for myotoxin *a*, appears to activate tissue  $\text{PLA}_2$  activity. Convulxin, which appears to activate a PI-hydrolyzing PLC, did not significantly affect lipid metabolism in our system, suggesting it may be specific for  $\text{PIP}_2$ . Thionin appears to be similar to CTX and melittin in action. Bothropstoxin has a unique  $\text{PLA}_2$  activity.

#### Effects of Altered Lipid Environment on PSNTX action

The PSNTXs more readily hydrolyze differentiated over nondifferentiate nerve cells and less readily hydrolyze muscle culture phospholipids, possibly due to differences in lipid composition. Radiolabeling the cell cultures with 18:0 revealed far less phospholipid hydrolysis than if the cells were radiolabeled with 18:2.

#### Penetration of toxins into inner leaflet of plasma membrane bilayer

The unique substrate preference exhibited by the PSNTXs and the inability of BSA to extract specific fatty acids liberated by their enzymatic activity strongly suggests that they penetrate the plasma membrane bilayer. The lack of dependence on extracellular calcium of the activation of cellular lipases by CTX and melittin supports an intracellular action of these toxins also.

#### Recommended Changes or Future Work to Better Address the Problem

The synaptosomal preparation, but only when employed with BSA, appeared to be a highly useful biochemical model for examining the mechanisms of the PSNTXs. However, it became obvious that the rat preparation is superior to the mouse preparation, as regards the wider range of toxins that were effective, and would be the model of choice for the snake venom PSNTXs.

The cell culture systems, while highly useful for the phospholipid hydrolysis studies, were not useful in the ACh release studies. Special attention must be paid to obtaining clones of cell lines that are functional in this respect.

We found it highly useful to add the *Naja naja kaouthia*  $\text{PLA}_2$  to the nonPSNTXs  $\text{PLA}_2$ s

toward the second half of the study. Future studies should probably include at least three nonPSNTXs PLA<sub>2</sub>s to get a better idea of the range of substrate specificity for these enzymes. Also, a minimum of five PSNTXs with widely varying toxicities (as we employed) should be used.

Apparently skeletal muscle cell cultures do not properly load the fluorescent Ca<sup>2+</sup> dyes. While we had some success in this area, the cells are overall not conducive for these types of studies. We have no suggestions as to better approaches, except perhaps to try different cell types. We were highly successful in the past with lymphocytes and epithelial cell lines.

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